The effects of D-penicillamine on platelet functions in rheumatoid arthritis

Jackson, Peter John

Award date:
1987

Awarding institution:
University of Bath

THE EFFECTS OF D-PENICILLAMINE ON PLATELET FUNCTIONS IN RHEUMATOID ARTHRITIS

Submitted by

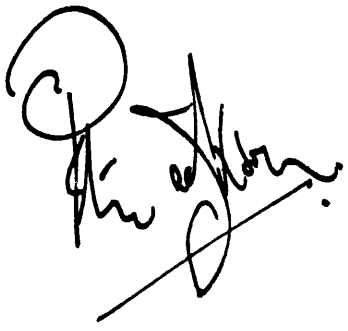
Peter John Jackson. M.Sc., F.I.M.L.S.,

for the Degree of Doctor of Philosophy

of the University of Bath.

1987

COPYRIGHT



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To Sonja, Alison & Emma

For their patience and encouragement

SUMMARY

Blood platelets are important cells in the inflammatory process and are useful for investigating receptor-linked mechanisms of activation and the effects of drugs.

A number of platelet functions have been investigated in groups of healthy individuals and patients with rheumatoid arthritis, before and during treatment with D-penicillamine. Evidence of abnormal platelet function was found in rheumatoid arthritis including, thrombocytosis, reduced platelet volume, increased platelet turnover, in particular, rheumatoid platelets showed enhanced sensitivity to aggregation induced by ADP, adrenaline and especially collagen. These data imply consumption of platelets during the rheumatoid disease process.

Treatment of rheumatoid patients with D-penicillamine brought about the abolition of the aggregatory response to adrenaline and reduced sensitivity to the other agonists. There was also a general trend towards normalisation in all platelet parameters measured. The loss of repsonse to adrenaline was found to be partly due to blockade of platelet $alpha_2$ adrenoceptors, and partly to another effect, possibly on the Gi coupling protein. The inhibition brought about by D-penicillamine on platelet aggregation was reversed by incubation with Dithiothreitol in vitro.

Thus, D-penicillamine may bring about some of its anti-rheumatic actions through effects on platelets, or by similar biochemical effects on other cell types active in rheumatoid disease.

ACKNOWLEDGEMENTS

I wish to thank Dr.N.D.Hall for his kind supervision, guidance and encouragement throughout the whole of this thesis.

I am very grateful to Dr.J.Woodland, Consultant Rheumatologist, Gloucester Royal Hospital, for the selection and supply of rheumatoid patients.

I would also like to express my gratitude to Dr.P.J.Kingston, Consultant Haematologist, Department of Clinical Pathology, Gloucester Royal Hospital, for providing facilities for this work, and his invaluable advice.

I am also indebted to the administration and members of Gloucester's Health Authority for allowing me to pursue this degree.

Finally, I owe grateful thanks to Janet Trigg and John Evans for their extreme patience and excellent word-processing skills in preparing this manuscript.

CONTENTS

CHAPTER ONE:

INTRODUCTION

1.1 General Introduction

An investigation into the mode of action of a drug in a pathological process requires some knowledge of the physiological and biochemical changes that have occurred in the system which differentiate it from the normal state. Conversely, investigations into proposed mechanisms of drug activity may lead to important information in the understanding of the pathological process.

1.2 The Inflammatory Process

Inflammation has been defined as a response of a tissue to sublethal injury (Houck, 1968) and such a general statement adequately summarizes the various forms in which the process may be manifested. Heat, redness, swelling, pain and loss of function, the cardinal signs of inflammation were first described by Celsus (30B.C.-A.D.38) and Galen (129-201A.D.). Regardless of the type of injury (traumatic, immunological, chemical or thermal) or the particular location of the tissue itself, the immediate event is that cells are adversely affected by the stimulus and an inflammatory reaction is initiated. Typical features of the reaction can be recognised at the tissue, cellular and subcellular level.

The events of the inflammatory process, outlined later, may follow a simultaneous rather than a sequential and orderly

pattern. The biochemistry and physiology of the inflammatory process is complex, and is still to be fully elucidated at many stages, but in vitro methods in correlation with in vivo observations appear to be the only way in which a true understanding of the underlying cause of the pathological inflammatory response and realisation of the mode of activity of anti-inflammatory drugs may be achieved.

The events of the inflammatory process have been the subject of numerous books, reviews and papers. Notable amongst them are the works of Zweifach, Grant & McCluskey, (1965); Spector & Willoughby, (1968); Forscher, (1968); Weiner & Piliero, (1970); Chayen & Bitensky, (1971); Willoughby, (1973); Zvaifler, (1973); Billingham & Davies, (1979); Zvaifler, (1979a); & Strober, (1981).

The primary effect of tissue injury is almost always manifested by a release of intracellular contents into an extracellular environment with a subsequent response in the micro-circulation. Cell derived small molecular mediators, such as histamine and 5-hydroxytryptamine (5HT), are liberated, primarily from tissue mast cells, to promote vasodilation and to increase the permeability of the venules and capillaries to circulating macromolecules and cells. These result in an accumulation of fluid at the injured site. The release of proteolytic and other digestive enzymes from lysosomes of either tissue cells e.g. synovial lining cells

in rheumatoid arthritis (Chayen & Bitensky, 1971) or invading polymorphonuclear leucoytes (Weissmann, 1967) is considered of prime importance in the development of the inflammatory response. Platelets are also capable of releasing 5-hydroxytryptamine and acid hydrolases which are mediators of inflammation, (Mills, Robb & Roberts, 1968).

As well as promoting tissue degradation, the lysosomal contents may also initiate the formation of other inflammatory mediators such as the kinins (Hopsu - Havu Makinen & Glenner, 1966); and prostaglandins (Anderson, Brocklehurst & Willis, 1971). These agents are considered chemotactic for polymorphonuclear leucocytes (Eisen, 1969; Kaley & Weiner, 1971) and, thus, might cause a concentration of active phagocytic cells in the inflammatory lesion (Higgs & Youlton, 1972). Furthermore, platelets are capable of stimulating chemotaxis via complement activation in a manner similar to leucocytes (Weksler & Coupal, 1973; Ruddy & Austen, 1975) and may therefore participate in early stages of inflammation. (Fig.1.1).

Platelet aggregation and fibrin deposition occur late in the inflammatory response causing thrombi formation, and stasis. Ischaemia, anoxia and acidosis of the wounded areas result, and tissue necrosis might follow. On the other hand macrophagic cells, fibroblasts and epithelial cells may

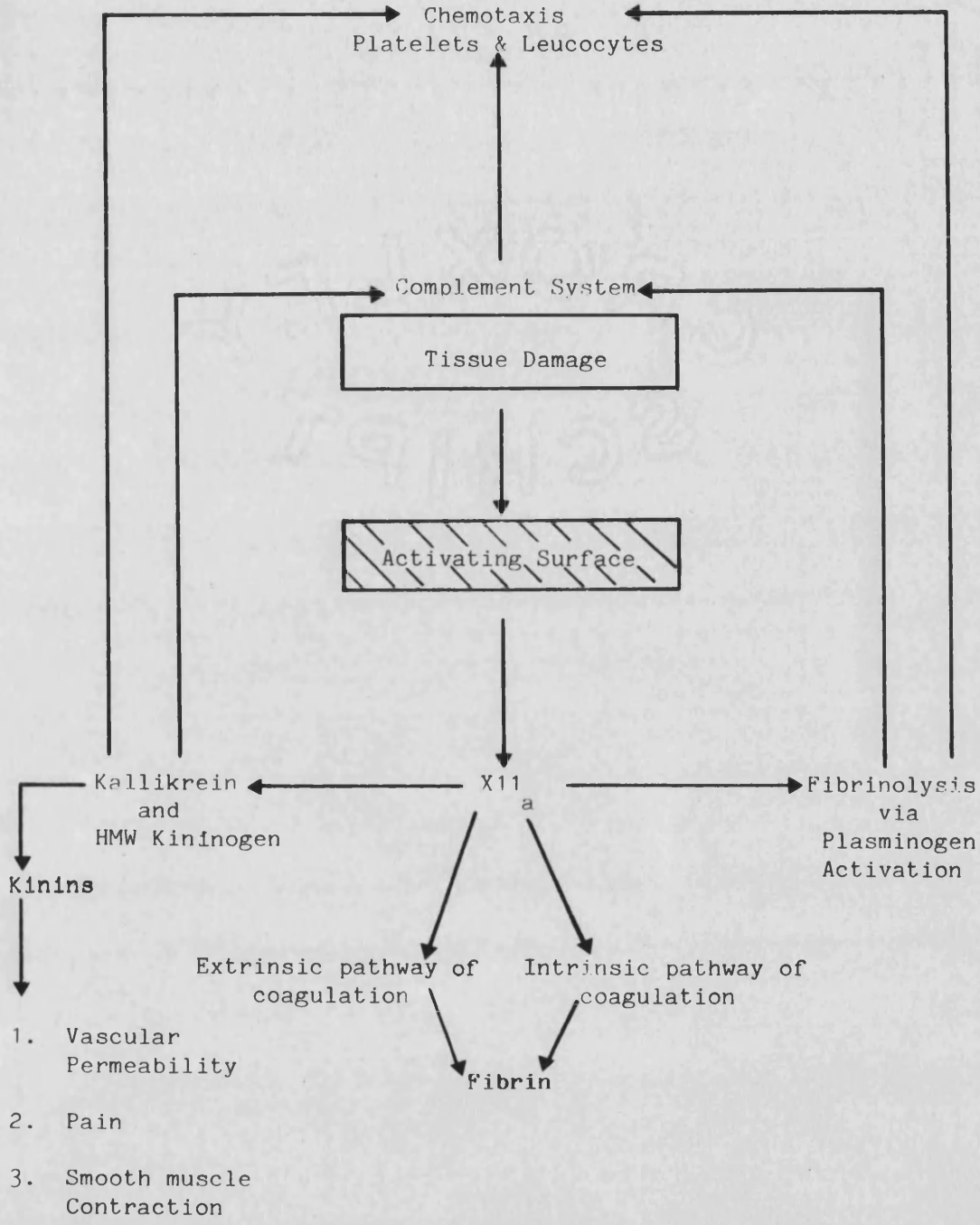


Fig. 1.1 Interaction between Platelets, Coagulation, Fibrinolytic and Inflammatory Mechanisms.

proliferate in the injured area to promote repair and restoration of function. This then represents the endpoint of the inflammatory reaction.

One of the most important aspects of the acute inflammatory response is the pathological development of chronic inflammation. Mechanisms of this progression are less well understood, but it has been widely accepted that the chronic response may result partly from the generation and release of autoantigens from the injured tissue, possibly involving a modification of host tissues by lysosomal enzymes.

Since the aetiology of virtually all inflammatory diseases remains unknown and cures for conditions such as rheumatoid arthritis remain elusive, the control of the inflammatory response with non-steroidal drugs and the use of second line drugs such as gold and D-penicillamine appear to be the most efficient therapeutic means of relieving much human suffering. Understanding the mode of action of these compounds might in turn lead to the development of more effective agents in the treatment of rheumatoid arthritis.

In this thesis I have examined D-penicillamine, a second line drug used in the treatment of rheumatoid arthritis, and its effects on blood platelets, the function of which may play a critical role in the self sustaining of the inflammatory process.

PART (1) RHEUMATOID ARTHRITIS

(A) The Pathogenesis of rheumatoid arthritis

1.1 Rheumatoid arthritis

Rheumatoid arthritis is a connective tissue disease of unknown aetiology which manifests itself both locally and systemically, but is characterised chiefly by an inflammatory erosive polyarthritis.

The typical picture of rheumatoid arthritis in a middle-aged or elderly woman with hands distorted and deviated to the ulnar side is familiar to almost everyone. This is, however, merely a common late result of a protean disease which may begin acutely or insidiously and affect almost any pattern of joints and show wide differences in its course and in its amenability to conventionally-used therapeutic measures.

There is a relatively rare but well defined childhood form of the disease in which the general symptoms of fever and weight loss are more conspicuous than in the adult. At all ages the most urgent symptoms are associated with synovitis of the joints leading to a variety of secondary local changes. There are inflammatory lesions of similar character in other synovial surfaces, bursae, tendon sheaths etc, and subcutaneous nodules are common.

1.2 Epidemiology

Recent epidemiological surveys in different populations have shown that the disease is common, affecting up to 3% of the adult population and apparently sparing no racial or ethnic group. Anyone at anytime can be stricken with the disease although statistically the incidence is 2-3 times higher in women than men with a peak incidence between the ages of 25-55 (Wood, 1977).

1.3 Anatomy

Glynn (1972) described the three outstanding anatomical features of rheumatoid arthritis to be (a) inflammation with progressive deformity of joints, (b) subcutaneous nodules and (c) vascular lesions.

A normal diarthrodial synovial joint consists of two bone ends capped with articular cartilage and joined by a fibrous capsule which is contiguous with the periosteum of the bones. The synovial membrane or "synovium" lines the capsule and is composed of a thin layer of cells, (Swinson & Swinburn, 1980). The lining layer of the synovium, the intima, is 1-3 cells in thickness and rests on a very vascular subintimal tissue which may be adipose, fibrous or areolar. Synovial cells or synoviocytes are responsible for the secretion of hyaluronic acid into the synovial fluid, the removal of solid particles

from the joint cavity and the activation of fibrinogen which is thought to prevent the accumulation of fibrin in the joint, (Fell, 1978). The intimal cells have been shown to be of two types - A cells which are phagocytic and resemble macrophages and B cells which are characterised by abundant, well developed rough surfaced endoplasmic reticulum, and are fibroblastic in appearance. The A cells are the predominant cell type. It is uncertain which types are responsible for specific functions, (Zvaifler, 1973; Fell, 1978).

In health, synovial fluid is thick, clear and viscous and contains hyaluronic acid; normal synovial fluid does not clot. Fibrinogen is absent but all the components of the fibrinolytic system necessary to produce free plasmin are present. Plasmin is the enzyme normally involved in the removal of fibrin. The volume of synovial fluid in a normal knee joint is quite small, being less than 2ml,(Zvaifler, 1973; Swinson & Swinburn, 1980).

1.4 Pathology

Early rheumatoid arthritis is characterised by swollen joints and a proliferative synovitis. Within the joint, excess connective tissue or "pannus" is produced by the proliferation of synovial lining cells with extensive vascularisation and infiltration of lymphocytes, plasma cells, polymorphonuclear leucocytes, platelets and macrophages (Harvey, 1978). The lining cells increase to a

depth of 6-10 cells (Zvaifler, 1973) and the pannus protrudes into the joint space. Harris (1976) suggested that irreversible joint destruction may begin with the degradation of articular cartilage which is mediated by the encroaching pannus. Chayen & Bitensky (1971) have demonstrated a high lysosome content in rheumatoid synovial lining cells and the presence of lysosomal enzymes in rheumatoid joints which may contribute to the inflammation. In studies of rheumatoid synovial membrane using electron microscopy, Kobayashi & Ziff (1973) identified perivascular accumulations rich in lymphocytes or plasma cells, or both types. Gaucher, Faure, Netter, Pourel & Duheille (1976) characterised cells of various sizes connected by intercellular bridges. Thus the rheumatoid synovial membrane presents a villous surface in comparison to the smooth flat surface of normal synovial membrane, with extensive cellular hyperplasia and hypertrophy.

The synovial fluid also varies considerably from normal fluid. The most striking differences are the total volume of fluid (200-400 ml in an active knee joint) and the ability of rheumatoid synovial fluid to clot, (Zvaifler, 1973). In rheumatoid arthritis synovial fluid protein and lipid levels are grossly elevated, while the haemolytic complement levels in rheumatoid patients are either normal or slightly increased.

It is obvious that the cells infiltrate the joint from the surrounding tissues or blood vessels. Zvaifler (1973) has demonstrated that the predominant cell in rheumatoid synovial fluids is a mature polymorphonuclear granulocyte, accounting for 75-90% of all nucleated cells. The remaining cells consist of 5-10% lymphocytes with some monocytes, platelets, macrophages and synovial lining cells, all of which may be involved in the pathogenesis of rheumatoid arthritis. (Denman, 1979).

Direct evidence of lymphocytes participating in this process was provided by Paulus, Machleder, Levine, Yu & MacDonald, (1977). Thoracic duct drainage produced a clinical improvement in rheumatoid patients and reinfusion of the cells induced exacerbation of disease activity. Support for these experiments from the use of lymphapheresis and lymphaplasmapheresis has also indicated some lymphocyte involvement (Tenenbaum, Urowitz, Keystone, Dwosa & Curtis, 1979; Karsh, Wright, Klippel, Decker, Deisseroth, & Flye, 1979).

Defects in the function of lymphocyte sub-populations may contribute to the pathogenesis of rheumatoid arthritis by several pathways. These include both helper and suppressor T lymphocytes, with defects leading to an inadequate response or to uncontrolled hyper-responsivness respectively (Slavin & Strober, 1981). Indeed, cellular abnormalities in

rheumatoid arthritis were shown to be predominantly within the T cell population and impaired T cell function has been reported in many studies, (Hall & Bacon, 1981). Lack of suppressor T cell activity from synovial tissue lymphocytes has been demonstrated, (Chattopadhyay, Chattopadhyay, Natvig, Michaelson & Mellbye, 1979). Abdou, Lindsley, Racela, Pascual & Hassanein, (1981) also demonstrated suppressor T cell dysfunction and the presence of anti-suppressor cell antibody in the peripheral blood of active early rheumatoid arthritis patients but not in inactive and late inactive disease.

Raised platelet counts have been reported in rheumatoid arthritis and a direct relationship noted between thrombocytosis and disease activity, (Selroos, 1972; Hutchinson, Davis & Jayson, 1976; Colli Maderna, Tremoli Colombo & Canesi, 1982). The interaction between platelets, rheumatoid factor and immune complexes, all implicated in rheumatoid arthritis, may represent a conceivable link with tissue lesions of inflammatory origin peculiar to the disease (Mueller-Eckardt & Luscher, 1968; Luscher, 1971; Margaretten & McKay, 1971; Pfueller & Cosgrove, 1980).
Hutchinson, Davis & Jayson (1976) also noted a significant correlation between thrombocytosis, vasculitis and other extra-articular manifestations and concluded that a compensatory over production, secondary to increased platelet consumption via intravascular coagulation may be

involved. This supports the work of Bennett, Eddie-Quartey & Holt (1972); Selroos & Wegelius (1973b); Selroos, Petterson, & Wegelius (1973c). Biochemical changes are common in rheumatoid arthritis (Kendall, 1977). However most of these are symptoms of an unhealthy state with little specificity for the condition.

1.5 Humoral Immunity

The presence of antibodies against the Fc region of autologous IgG (Rheumatoid Factor) in the serum of approximately 80% of patients with rheumatoid arthritis has been well documented for many years, (Waaler, 1940; Rose, Ragan, Pearce & Lipman, 1948); and has been extensively reviewed (Zvaifler, 1973; Johnson & Faulk, W.P, 1976; Ziff, 1980).

Classical IgM rheumatoid factor (IgMRF) led to the definition of seropositive and seronegative rheumatoid arthritis. No correlation was found between IgMRF and raised immunoglobulin levels in rheumatoid patients, (Barden, Mullinay & Waller, 1967). More recently IgG rheumatoid factor has been demonstrated in both seropositive and seronegative rheumatoid patients but not in other seronegative rheumatoid diseases (Allen, Elson, Scott, Bacon & Bucknall, 1981). High levels are associated with

rheumatoid vasculitis and IgA rheumatoid factors have also been described (Johnson & Faulk,W.P, 1976; March, Reeback, Holborow & Coombs, 1981).

Rheumatoid factors could contribute to the presence of circulating antigen antibody complexes and rheumatoid arthritis is often termed an "Immune Complex Disease". Complexes can interfere with Fc receptor meditated functions and, when deposited in the tissues , may have pathological effects. Glomerulonephritis is probably immune complex induced (Wiggins & Cochrane, 1981), although this is rare in rheumatoid arthritis.

The rheumatoid synovial membrane is known to synthesise immunoglobulin of mainly IgG class (Smiley, Sachs & Ziff, 1968). IgG and IgG complexes can be synthesised intracellularly and not secreted. Approximately 50% of plasma cells are synthesising IgG (Natvig & Munthe, 1975). Immune complexes containing IgM, IgG and complement have been detected in synovial fluids, (Zvaifler, 1974). Britton & Schur (1971) described intracytoplasmic inclusions of immunoglobulins and complement in synovial fluid leucocytes, they suggested that immune complexes within the synovial fluid fix complement and through activation of C3 are phagocytosed by synovial fluid leucocytes. This may provide an explanation for the low levels of complement found in the joints of rheumatoid patients (Winchester, Agnello & Kunkel,

1969; Winchester, Agnello & Kunkel, 1970; Ruddy & Austen, 1975).

Other autoantibodies have been demonstrated in rheumatoid arthritis e.g.anti-nuclear antibodies (ANA) or anti-nuclear factors (ANF) (Holborow, 1979). These antibodies are found mainly in patients with systemic lupus erythematosus (SLE) although they are sometimes found in rheumatoid patients. Grennan, Sloane, Behan & Dick (1977) showed that rheumatoid patients with antibodies to double stranded DNA are more likely to have severe disease and extra-articular complications. Anti-collagen antibodies have been detected in both rheumatoid sera and synovial fluid, and collagenase activity has been proven in synovial membranes and fluid (Steffen, 1980).

1.6 Aetiology

The search for a primary antigen in rheumatoid arthritis has so far proved negative. It was originally postulated that the "Rheumatoid Factor" was the cause of rheumatoid arthritis but the discovery of high titres in other diseases dispelled this assumption (Zvaifler, 1973; Johnson & Faulk,W.P, 1976; Carson, Lawrance, Catalano, Vaughan & Abraham, 1977).

Using the human leucocyte migration inhibition test (LMT) some researchers have demonstrated a specific response by

rheumatoid patients to synovial eluates (Bacon, Cracchiolo, Bluestone, & Goldberg, 1973). Other investigations however, using very large numbers of patients have shown this effect to be non-specific (Morgan, Hall Collins, & Bacon, 1980).

An infectious agent has been implicated as the instigator of rheumatoid arthritis since many immunopathological features resembling those of connective tissue disease are associated with infections. It is known that arthritis complicates many bacterial and viral infections such as rubella, although it is nearly always short lived and non erosive. A wide variety of autoantibodies are produced in some infectious disease e.g. subacute bacterial endocarditis and anti IgG can neutralise infectious complexes of antigen found in viruses such as herpes simplex. Thirdly, virus infection has been implicated in some degenerative diseases of the central nervous system i.e. slow virus infection and influenza virus has been shown to precede Goodpastures syndrome :- an autoantibody induced glomerulonephritis in which antibodies to glomerular basement membrane are produced . There is commonly an immune complex nephritis in infection including sub acute endocarditis. This subject is reviewed by Denman (1975) and Denman, Appleford, Imrie, Kinsley, Pelton & Schnitzer, (1977). These authors favour a viral aetiology for rheumatoid arthritis but this is not proven.

Following many viral infections, viral antigens are expressed on the surface of the infected cells and the viral genome may become integrated into host DNA, thus persisting in the host cell. Norval & Smith (1979), found no evidence of viral genomes in the DNA and RNA of synovial fibroblasts, synovial membranes or peripheral blood lymphocytes from patients with rheumatoid arthritis or other joint conditions.

Appleford & Denman (1979a) isolated lymphocytes from the peripheral blood and synovial fluid of rheumatoid patients and showed that only peripheral blood lymphocytes support the growth of Herpes Simplex Virus (HSV) . The same workers showed that the T lymphocytes isolated from synovial fluid could support the growth of HSV but the readdition of non-T lymphocytes again caused non permissiveness. However, if synovial fluid non-T lymphocytes were added to autologous peripheral blood T cells no such effect was noted and the virus grew normally. Removal of glass adherent cells from non-T Synovial fluid lymphocytes did not remove inhibition. The authors suggest that synovial non-T cells may be producing anti viral factors (Appleford & Denman, 1979b). Other investigators suggesting a viral aetiology for rheumatoid arthritis described the presence of a serum antibody in rheumatoid patients which is reactive with a soluble nuclear antigen extracted from human lyphoblastoid lines infected with Epstein Barr Virus (EBV), but not those

infected with other Herpes type viruses. Thus rheumatoid arthritis associated nuclear antigen (RANA) appears to be distinct from Epstein Barr Nuclear Antigen (E.B.N.A.) (Alspaugh, Jensen, Rabin & Tan, 1978). A high frequency of anti-RANA antibody was shown to occur in both seropositive and seronegative rheumatoid arthritis . Normal controls and patients with other arthritic conditions had low levels of antibody (Ng, Brown, Perry & Holborow, 1980). Catalano, Carson, Niederman, Feorino & Vaughan (1980) studied healthy individuals and showed that those having antibodies to Viral Capsid Antigen (VCA) which is expressed during EBV infection, also had anti- RANA antibodies, while anti-VCA negative individuals were anti-RANA negative. They therefore concluded that anti-RANA antibodies are produced only after EBV infection. Ferrell, Aitcheson, Pearson & Tan, (1981) confirmed the high incidence of anti-RANA antibodies in RA patients and its distinction from anti EBNA. Other EBV-associated antibodies were also detected. Rheumatoid arthritis suppressor cells are poor regulators of EBV outgrowth (Depper & Zvaifler, 1981) but this effect is not specific for rheumatoid arthritis .

1.7 Genetic Factors

Rheumatoid arthritis is a disease of relatively recent origin (Bywaters, 1975). It has been suggested that enviromental factors may operate in genetically predisposed individuals

to promote development of the disease. Interest in the role of genetic factors in rheumatoid arthritis was generated through the work of Brewerton, Caffrey, Hart, James, Nicholls & Sturrock (1973) Schlosstein, Terasaki, Bluestone & Pearson (1973) who demonstrated a very strong association between Ankylosing Spondylitis and the presence of an Histocompatibility Antigen (HLA-B27). The HLA antigen DR4 has been found in a higher proportion of rheumatoid patients than in healthy controls (Panayi & Wooley, 1977; Stastny, 1978; Gibofsky, Winchester, Pafarroyo, Fotino & Kunkel, 1978; Stastny & Fink, 1979). However, Jones, Jacoby, Johnson, Phua & Welsh (1983) suggested that HLA-DR4 is a marker of disease severity in rheumatoid arthritis rather than of suceptibility to arthritis.

Hence it appears that rheumatoid arthritis may be due to the exposure of individuals with a particular genetic constitution including HLA-DR4 to an infectious agent. The illness may result from an inappropriate immune response to a ubiquitous agent, or conversely, the individual who develops rheumatoid arthritis may be less able to withstand infection by an uncommon pathogen, (Zvaifler, 1979a).

(B) Treatment of Rheumatoid Arthritis

1.1 First line drugs

a. Non-steroidal anti-inflammatory drugs (NSAIDs)

The major classes of non steroidal anti-inflammatory drugs are (a) salicylic acids e.g. aspirin, (b) carboxylic acids:- 1. acetic acids e.g. indomethacin, 2. propionic acids e.g. ibuprofen, 3. fenamic acids, mefenamic acid, (c) enolic acids:- 1. pyrazolones e.g. phenylbutazone 2. oxicams e.g. piroxicam (Nickander, McMahon & Ridolfo, 1979).

The NSAIDs can provide symptomatic relief, i.e. relief of pain, stiffness and swelling but are not thought to have any influence on the actual disease process (Vane, 1971; Smith & Willis, 1971; and Hart, Huskisson & Ansell, 1982). Vane (1971) was the first worker to show that NSAIDs such as aspirin and indomethacin inhibited prostaglandin synthesis by blocking the cyclo-oxygenase pathway. However, their potencies as cyclo-oxygenase inhibitors vary widely (Crook, Collins, Bacon & Chan, 1976). It is now thought that some of the NSAIDs may have specific effects upon the lipoxygenase pathway as well. The lipoxygenase pathway releases potent inflammogenic components from platelets and leucocytes (Moncada & Vane, 1979). Blockade of the cyclo-oxygenase pathway could result in increased activity of the lipoxygenase pathway and thus enhance inflammation, (Humes,

Winter, Sadowski & Kuehl, 1981), prostaglandin E_2 and prostacyclin are the chief components of this kind. All NSAIDs exhibit not only analgesic, anti-inflammatory and anti-pyretic actions but also characteristic gastrointestinal side effects, possibly related to their common modes of action.

The effect of NSAIDs on prostaglandin production is now well documented, although other potential sites of action are numerous e.g. kinins, leukotrienes and oxygen radicals. Indeed non steroidal anti-inflammatory drugs have been shown to be inhibitors of chemiluminescence generated by H_2O_2 - myeloperoxidase Cl, the major cytoxic system of neutrophils (Pekoe, Van-Dyke, Mengoli, Peden & English, 1982). Thus part of the inflammatory effect of NSAIDs may be due to interference with the oxidative metabolism of neutrophils.

1.2 Second line drugs

Second line drugs are "disease modifying" or "slow acting" which are used generally in patients with severe active rheumatoid arthritis only after an adequate trial of first line NSAIDs has failed to relieve symptoms satisfactorily. Gold and penicillamine are examples of these and actually suppress disease activity in some patients with rheumatoid arthritis, slowing down the rate of progression of joint damage. However they both have potentially serious side effects.

a. Gold

Gold compounds are actually administered intramuscularly as aqueous sodium aurothiomalate or as an oil-based aurothioglucose. The mode of action of gold in rheumatoid arthritis is not known, but it may possibly act via thiol groups on the surface of cell membranes.

Gold has been used in the treatment of rheumatoid arthritis for over fifty years (Forestier, 1929). Its efficacy is well established in rheumatoid arthritis when disease activity cannot be controlled with NSAIDs (Empire Rheumatism Council, 1960; and 1961; American Rheumatism Association, 1973; Walz, DiMartino & Sutton, 1974). The results of these studies were confirmed by Sigler, Bluhm, Duncan, Sharp, Ensign & McCrum (1974). In a double blind trial they extended these findings to positive radiological evidence, proving that gold therapy significantly slowed the progression of disease activity in the joints of rheumatoid patients.

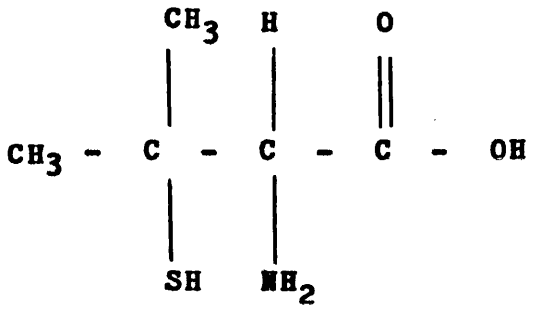
Clinical improvements are seen in 60-70% of patients with reductions in circulating immune complexes in advance of rheumatoid factor decrease (Nineham, Hay, Male & Roitt, Young & Perumal, 1979). The majority of gold injected is sequestered throughout the body tissues and has been demonstrated in lysosomes of macrophages and lining cells of the synovium, where it may inhibit the release of lysosomal

enzymes, (Weissmann, 1972). A variety of enzyme systems are influenced by gold, particularly those requiring free SH groups (Zvaifler, 1979b). Comparisons of thiomalate with D-penicillamine, also an effective drug in rheumatoid arthritis, have led to the intriguing proposal that they may act by a common mechanism. Both are sulphydryl compounds (Munthe, Jellum & Aaseth, 1979) and there are similarities in their clinical effects - slow response, prolonged effectiveness and their side effects.

b. D-penicillamine (D-pen)

Penicillamine was discovered in acid penicillin hydrolysates (Abraham, Baker, Chain, Florey Holliday & Robinson, 1942); and was shown to be a fundamental part of the penicillin molecule. It was later given the name penicillamine because of its close relationship to penicillin and because it was an amino acid (Abraham, Chain, Baker & Robinson, 1943). The presence of a sulphydryl group on the beta carbon led to the structural formula of penicillamine (Chain, 1949). Much work was carried out on D-pen treatment of patients with Wilson's Disease due to its copper-chelating properties (Walshe, 1956). Deutsch & Morton (1957) observed that certain thiols dissociated macroglobulins, and this ultimately led to the considerable and subsequent study of the treatment of rheumatoid arthritis with penicillamine (Jaffe, 1962; Jaffe, 1963; Jaffe, 1965; Multicentre Trial Group, 1973; & Jaffe, 1979).

STRUCTURE OF PENICILLAMINE



Mode of Action

Its mode of action in rheumatoid arthritis remains unknown, but it may act upon disulphide bonds and thiol groups, on cell membranes or on metallo-enzymes. The usual initial dose of penicillamine is 125mg/day, increasing by 125mg in steps every month until there is a clinical response. Some patients who fail to respond benefit from higher doses, occasionally up to 1,000mg/day. However, the incidence of side-effects increases rapidly at this dose level. Side effects of penicillamine are similar to those of gold and include thrombocytopenia, neutropenia and gastric irritation. Proteinuria, taste loss and skin rashes are common and may occur at any stage of therapy.

Penicillamine is an amino acid with thiol, amino and carboxyl functional groups. Three types of reaction play an important role in explaining the pharmacological and possible therapeutic actions of this drug:-

(a) Thiol-disulphide exchange, (b) Thiazolidine formation,

(c) Metal chelation (Friedman 1977; Nimni 1977; Sarkar, Sass-Kortsak, Clarke, Laurie & Wei, 1977).

In human blood, penicillamine forms complexes with various metals including lead and zinc (Balogh, El-Ghobarey, Fell, Brown, Dunlop & Dick, 1980) but its complexes with copper may have a more important role in changing or reducing inflammation in rheumatoid arthritis (Jaffe 1979; Munthe, Jellum & Aaseth, 1979; Greenwald, 1981; Clark, 1983; Davis, 1984; Staite, Zoschke & Messner, 1984). The D-pen-copper complex possesses superoxide dismutase activity, in that it removes superoxide radicals generated by macrophages by catalysing the reaction to hydrogen peroxide. The hydrogen peroxide liberated is itself harmful to cell membranes and this is further removed by the enzyme catalase, (Greenwald 1981; Clark, 1983; Staite, Zoschke & Messner, 1984).

The reactivity of penicillamine with natural thiol compounds is not necessarily restricted to mixed disulphide formation with extracellular cysteine or with circulating macroglobulins like IgM. The drug may also react with free SH groups in other proteins such as serum albumin, resulting in the displacement of bound low molecular weight compounds (Stanworth, 1984). Hall & Gillan (1979) have shown that penicillamine enhances SH-SS exchange reactions and Gerber (1978) reported that D-pen inhibits the SH-dependent heat aggregation of IgG in vitro and possibly the formation of auto IgG aggregates in vivo.

Many observations have been made on the long term action of

penicillamine on plasma immunoglobulins and rheumatoid factors. Immunoglobulin levels of all classes are affected by penicillamine and IgA deficiency has resulted in some cases (Hjalmarson, Hanson & Nibson, 1977;Stanworth, Johns Williamson, Shadforth, Felix-Davies & Thompson, 1977; Mbuyi-Muamba, Stevens & Decqueker, 1981; Gul, Corke, Huskisson & Holborow, 1984). Stanworth & Hunneyball (1979) have linked the tendency of penicillamine induced Class A Immunoglobulinaemia to possession of the HLA A3B40 haplotype.

Penicillamine responders who are initially classical seropositive, will demonstrate a fall in serum IgMRF levels, (Gul, Corke, Huskisson & Holborow, 1984). It is possible that the IgG complex becomes a stimulus to IgMRheumatoid factor production because of sulphydryl-disulphide rearrangements within the IgG molecule. Penicillamine by cross-linking with the sulphydryl groups, might prevent these rearrangements and so IgM antibodies to IgG are no longer produced (Pritchard & Nuki, 1978; Wollheim, 1981).

Lipsky(1981) proposed that penicillamine might exert an immunosuppressive action and inhibit the function of cells involved in humoral immune responses.
Penicillamine, in the presence of copper, has been shown to inhibit the proliferative response of human peripheral blood T cells to non-specific phytomitogens (Lipsky & Ziff, 1978).

In these experiments, the T cell function appeared to be specifically inhibited while the monocyte accessory function was not affected. Later, Lipsky & Ziff (1982) found that penicillamine also inhibited the capacity of peripheral blood mononuclear cells to generate immunoglobulin-secreting cells in response to in vitro stimulation with T cell-dependent polyclonal B cell activators. Again, inhibition was observed only when cells were incubated with penicillamine in the presence of copper ions. Other compounds such as 2-mercaptoproprionylglycine and 5-thiopyridoxine also inhibited responsiveness in the presence of copper in this system (Lipsky & Ziff, 1982). The observation that pre-incubation with penicillamine and copper ions did not alter the capacity of B cells to form immunoglobulin-secreting cells after appropriate stimulation, but rather diminished helper T cell activity, implies that the action of penicillamine and other thiols results from a selective inhibition of T cell function, (Lipsky, 1981; Lipsky, 1984).

In addition to these effects, wide ranging actions of penicillamine have been reported, reduced levels of complement that may demonstrate an effect of the drug on the macrophage (Pritchard & Nuki, 1978; Munthe, Jellum & Aaseth, 1979); an effect on neutrophils possibly due to the -COOH group on the penicillamine molecule activating granulocytic chemotactic response (Davis, 1984) and the stabilisation of lysosomal membranes and increases of lysyl

oxidase activity, thereby leading to increased repair of collagen, (Munthe, Jellum & Aaseth, 1979; Davis, 1984). Thrombocytopenia is a fairly common side effect of penicillamine therapy and it has been estimated that between 8 and 31% of patients will show a fall in their platelet count (Camp, 1981; Lyle, 1983; Thomas, Gallus, Brooks, Tampi, Geddes & Hill, 1984). This is of great interest in that a very large proportion of untreated rheumatoid patients present initially with a thrombocytosis. The mechanism causing a reduction in total platelet count remains unknown. It has been suggested it may be an auto immune reaction (Pegels, Ament, Van Der Plass Den Dalen, Van Dem Borne & Feltkamp, 1982). Conversely, it has been shown that penicillamine alters platelet production rather than damaging existing platelets, (Thomas, Gallus, Brooks, Tampi, Geddes & Hill, 1984).

The only conclusion that can be drawn about the mode of action of penicillamine is that it is very complex. It may not be simply explained by immunomodulation; but, all three types of reaction outlined may play a role in its pharmacological actions.

1.3 Third line drugs

a. Corticosteroids

There is no convincing evidence that corticosteroids modify

the course of rheumatoid disease, and they are now used with caution and in defined limited circumstances. Non-specific immunosuppression results in the risk of infection as well as vascular necrosis, osteoporosis and vertebral collapse. However, these side effects are only relevant to high doses.

Mode of Action

Corticosteroids are the most powerful anti-inflammatory drugs available and their pharmacological effects are extensive. They have wide ranging effects on all aspects of humoral and cellular immunity and the reticuloendothelial system, with no specificity for those cells involved in pathogenesis. Corticosteroids inhibit vascular responses to chemical mediators preventing increased permeability and vasodilation seen in acute inflammation. The accumulation of platelets, neutrophils, monocytes, macrophages and T and B lymphocytes is inhibited (Fauci, Dale & Balow, 1976; Parrillo & Fauci, 1979) and antibody production is reduced and clearance of immune complexes by the reticulo-endothelial system is lowered. Suppression of arachidonic acid release and resultant lack of synthesis of the cyclo-oxygenase and lipoxygenase products such as leukotreine B4 (LT B4) and interference with the generation of platelet aggregating factor (PAF), results in an overall reduction in pro-inflammatory activity, (Lewis & Piper, 1975; Hirata, Schiffmann, Venkatasubramanian, Saloman & Axelrod, 1980).

These effects on mediator production are secondary to inhibition of phospholipase A_2 by a steroid-induced protein, lipocortin (Flower, 1984; Flower, 1985).

The effect on the production of the inflammatory mediators may help to explain the potency of corticosteroids compared to other treatment. Important actions on carbohydrate, lipid and protein metabolism are reflected in the clinical appearance of patients receiving steroid therapy. The growth of fibroblasts is inhibited by glucocorticoids resulting in thinning of the skin, loss of protein, muscle proteolysis and very poor wound healing.
Since synovial fluid cells have been shown to concentrate steroids from solution at relatively low concentrations, the ability of the drugs to stabilize lysosomal membranes and prevent enzyme release may be an important feature, (Lewis & Day, 1972). This however is unlikely to be a definite mechanism of action.

(b) Cytotoxic agents

The immunosuppressive agents azathioprine, cyclophosphamide methotrexate and chlorambucil can suppress both immune and non immune inflammation in experimental models and it has been suggested that this may be their primary mode of action in rheumatoid arthritis. However, evidence of immune suppression in rheumatoid patients on these drugs is

conflicting. Cell mediated responses have been reported as depressed or unaltered (Currey, 1978), although azathioprine (Hurd & Ziff, 1974) and cyclophosphamide, (Campbell, Skinner, McLennan, Hersey, Waller, Wood, Jewell & Truelove, 1976) are known to deplete lymphocyte populations. High doses primarily cause reduction of B lymphocytes, plasma cells and antibody productivity, whereas smaller doses can actually enhance the production of antibodies and autoantibodies in experimental models by a differential effect on T suppressor lymphocytes, (Chanmougan & Schwartz, 1966). All four drugs have severe toxic effects and long term treatment is prevented by the anxiety over late toxicity e.g. the likelihood of developing malignancy, especially Hodgkins lymphoma, mutagenesis, bone marrow and renal toxicity, (Bertino, Hurd, Decker & Steinberg, 1973).

1.4 Experimental therapies

(a) Plasmapheresis

Researchers have attempted to modulate the immune system in rheumatoid arthritis by plasmapheresis. This has been beneficial in some immune complex diseases e.g. systemic lupus erythematosus (Hay, Nineham, Male, Roitt & Parry,1979a; Verrier-Jones, Cumming, Bacon, Evers, Fraser, Bothamley, Tribe, Davis & Hughes, 1979). Goldman, Casey, McIlwain, Kirby, Wilson & Miller (1979) and Bacon (1979) have

treated vasculitis in rheumatoid arthritis by plasmapheresis. This resulted in a fall in circulating immune complex levels but symptoms reappeared when plasmapheresis was discontinued and no improvement was found in five other patients.

(b) Total Lymphoid Irradiation

Irradiation of total lymphoid tissue has been tried in several uncontrolled studies and in some instances symptoms have improved, (Kotzin, Strober, Engleman, Calin, Hoppe, Kansas, Terrell & Kaplan, 1981). However the results of controlled studies are awaited and since there is a high morbidity risk from such therapy it should be confined to specific centres conducting carefully controlled research and only involving patients where all conventional therapy has failed.

1.5 Alternative Medicine

Many homeopathic and diet regimes have been postulated in treating rheumatoid arthritis but there is little evidence that they have any beneficial effect in controlling the disease. However, Heptinstall, White, Williamson & Mitchell (1985) and Heptinstall (personal communication) have examined extracts of feverfew (**tanacetum parthenium**), a herbal remedy used since ancient times as a remedy for fever, arthritis and migraine. These extracts were found to

inhibit secretory activity in blood platelets and polymorphonuclear leucocytes. Release of serotonin from platelets induced by various aggregating agents (ADP), adrenaline, sodium arachidonate, collagen and U46619 was inhibited. Platelet aggregation was consistently inhibited but thromboxane synthesis was not. Feverfew also inhibited release of vitamin B12 binding protein from polymorphonuclear leucocytes induced by secretagogues N-formyl-methionyl-leucyl-phenylalanine, sodium arachidonate and zymosan-activated serum. Feverfew did not inhibit the secretion induced in platelets or polymorphonuclear leucocytes by the calcium ionophore A23187.

The pattern of the effects of feverfew extracts on platelets is different from that obtained with other inhibitors of platelet aggregation and the effect on polymorphonuclear leucocytes more pronounced than that which has been obtained with very high concentrations of non-steroidal anti-inflammatory agents. These findings are extremely interesting and may relate to the inflammatory process and the claimed benefit for feverfew treatment in rheumatoid arthritis.

PART (II) BLOOD PLATELETS

(A) Introduction

1.1 General Introduction

The mechanisms of physiological haemostasis, pathological thrombosis and inflammation have received much attention in recent years. These processes involve the interaction of a number of factors including platelets and (a) Neutrophils, Lymphocytes and Monocytes, (b) The Blood Coagulation system (c) The Fibrinolytic System, (d) The Complement System and (e) Vascular contraction and altered haemodynamic mechanisms.

1.2 History

In 1842 George Gulliver and William Addison provided the earliest drawing of cells that appear to be platelets. Max Schultze (1865) using a method for keeping the blood cells warm during microscopic examinations observed that platelets could coalesce into granular masses. In the following year Booth and Himmelburgh (1866) introduced the concept that platelets play an important primary role in the formation of the haemostatic plug. Their experiments demonstrated that on cut carotid arteries in the dog, the platelets plugged the gap in the vessel wall, formed aggregates and fused into one

another. It was William Osler in 1874 who described resting platelets and their ability to aggregate and clump which are consistent with today's current concepts. He suggested, however, that the cells he observed were possibly types of bacteria. Platelet clumping was also noted by Georges Hayem (1878) but he failed to understand their significance and concluded that platelets served as precursors of erythrocytes. Julius Bizzozero (1882) made several remarkable contributions by accurately illustrating the appearance of activated platelets, describing their adhesive properties and recognizing that these cells participated in blood coagulation and thrombosis. He had previously reported in 1868 that blood cells originated in the bone marrow.

The functions of platelets were clearly demonstrated by Karl Eberth and Curt Schimmelbusch in 1886. They described changes in the morphology of platelets that had come in contact with foreign surfaces or damaged vessels as "viscous metamorphosis" a term which is still used in medical practice. J. Homer Wright (1906) used his famous stain to study bone marrow cells and established that platelets originated in megakaryocytes.

Despite these elegant scientific observations and studies made in the nineteenth century, the platelet and its physiological significance were not appreciated during the

first half of the twentieth century. Unfortunately interest turned from the platelet to other components of blood coagulation, in particular those related to the formation of fibrin clot.

Current appreciation of the biology of platelets and their integral role in haemostasis and inflammation can be attributed to the talent and dedicated efforts of a number of investigators over the last twenty five years. Of these, Arvid Hellem is chiefly noted for developing innovations in methodology and for devising a useful quantitative test of platelet function:- Platelet adhesiveness (Hellem, 1960). This test undoubtedly stimulated the development of the method for studying platelet aggregation which is now an important diagnostic and investigative tool. Hellem applied the test to demonstrate that erythrocytes contained a substance that caused platelets to become 'sticky' and to adhere to glass and to each other. He named this substance "Factor R" and characterized it as a small acid molecule that was heat stable.

Hellem participated in further investigations (Gaarder, Jonsen, Laland, Hellem & Owren, 1961) and identified "Factor R" to be adenosine diphosphate (ADP). It is accepted today that platelets contain and secrete ADP and that ADP is pivotal for platelet physiology. The enormous proliferation of research during the last decade as well as the understanding

of the role of blood platelets both in health and disease, in vessel-wall injury, arterial and venous thrombosis, atherosclerosis and antiplatelet therapy, can in large part be related to Hellem's contributions.

(B) Thrombokinetics

1.1 Platelet Production

Platelets are formed in the bone marrow by fragmentation of the cytoplasm from giant precursor cells (megakaryocytes). Although megakaryocyte production is normally confined to the bone marrow it has been known for many years that megakaryocytes are present in large numbers in the lungs. There is evidence to suggest that the pulmonary megakaryocytes originate from the bone marrow and are carried to and retained in the lungs via the venous return (Kaufman, Airo, Pollack, Crosby & Doberneck, 1965). The contribution of the pulmonary megakaryocytes to the body platelet pool has been calculated to be as much as 7-17% (Kaufman, Airo, Pollack, Crosby & Doberneck, 1965).

Till and McCulloch (1961) showed that the megakaryocytes share a common precursor with the myeloid and erythroid series the pluripotent stem cell. They designated this cell CFU-S (spleen colony forming unit). In these now classic experiments, mice receiving lethal marrow irradiation and

injected with normal bone marrow cells showed macroscopic colonies on the splenic surface seven to ten days after transplantation, the number of colonies being proportional to the number of marrow cells injected. If these excrescences were dissected out and stained, haematopoietic colonies containing megakaryocytes, granulocytes, macrophages and erythroid cells were observed. Studies employing genetic markers have shown that each cell of an individual spleen colony arises from a single cell, thus the colony is a clone (Becker, McCulloch & Till, 1963). If the colonies are removed, dispersed into a suspension and subsequently injected into another group of recipients given lethal marrow irradiation, new splenic colonies appear, (Siminovitch, McCulloch & Till, 1963).

These experiments indicate that there are single cells which have the capacity for both self renewal and differentiation. The nuclei of the megakaryocytes are multilobed and exhibit polyploidy, the lobulated mass contains 2, 4, 8, 16, or 32 times normal diploid content of DNA. They have a unique pattern of cellular motivation in that, although DNA synthesis is completed at an early stage cells appear to undergo a series of nuclear divisions with a progressive increase in the volume of cytoplasm but without cell division. The earliest recognisable type of megakaryocyte found in the marrow has a nucleus which has undergone three divisions (8n cell). The nuclei of the megakaryocyte again divide several times to form 16n, 32n and then a 64n cell with

all the nuclei in a single megakaryocyte dividing synchronously.(Fig.1.2). Successive nuclear division results in an increase in cytoplasmic volume, which in turn increases the number of platelets formed. One single megakaryocyte is calculated to produce at least 2,000 platelets.

Formation of blood platelets in the bone marrow does not appear to be by a simple shedding or disintegration but rather by projection of the cytoplasm through the sinusoidal endothelium. It has been suggested that newly produced platelets are larger and more dense than those that have aged in the circulation (Karpatkin, 1969a & Karpatkin, 1969b). However, the majority of evidence supports the view that platelet volume and density are independent of age and are determined by the stage of megakaryocyte development (Paulus, Breton-Gorius, Kinet-Denoel & Boniver, 1974; Penington, Streatfield & Roxburgh, 1976; Corash & Shafer, 1982).

1.2 Regulation of Platelet Production

The blood platelets in man circulate as flattened cytoplasmic discs with a mean platelet volume (MPV) of 9.5 femtolitres in a concentration of 250 x 10^9/l (range 150 - 400 x 10^9/l). Although there is considerable interspecies variation (concentration of rodent platelets is four times that of

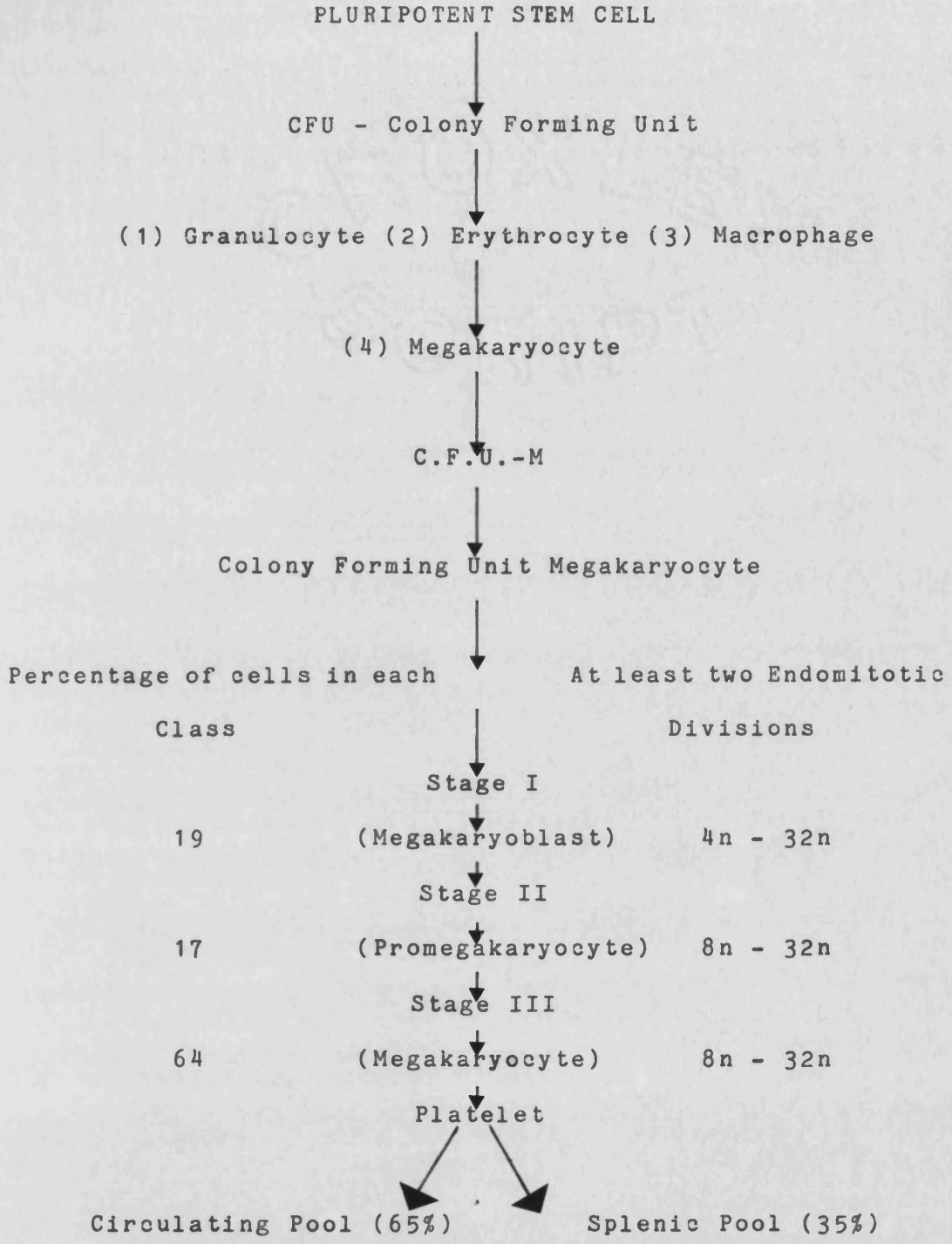


Fig.1.2 Schematic model of platelet production.

man) the intraspecies variation in the platelet count is minor and the day-to-day individual variation is minimal. The maintenance of the platelet count within such narrow boundaries, together with the initiation of compensatory changes in the megakaryocytic lineage subsequent to perturbations in the count, indicate that platelet production is a regulated process. It would appear that a regulatory hormone analogous to erythropoietin named "thrombopoietin" (Evatt & Levin, 1969) is responsible, mediating its effects by (a) increasing the number of megakaryocytes formed from precursor cells and (b) increasing the rate of both cytoplasmic maturation and platelet release. The site of synthesis of thrombopoietin appears to be the kidney and liver.

1.3 Platelet Kinetics

After their release from the bone marrow, the life span of the platelet is regulated by a very finite internal metabolism and its dynamic interaction with the vascular endothelium, the inflammatory response, and the coagulation and fibrinolytic systems.

The total body platelet mass is split between the splenic pool (approx 30%) and the blood circulation (approx 70%). Platelet survival studies using various isotopes indicate a survival time in the circulation of between 10 - 14 days (Harker & Finch, 1969) whilst the workers using a non radio-isotope

technique such as aspirin acetylation of the platelet enzyme cyclo-oxygenase gave a figure of 8 - 11 days (Harker, 1968). The spleen has an effect on the platelet distribution by sequesting approximately 30% of labelled platelets into the splenic cords of the red pulp. These can be exchanged freely with circulating platelets.

(C) Platelet Structure

1.1 Structure

The circulatory platelets in man are non-nucleated bodies of discoid shape measuring on average 2 - 4μm in length and 1um in thickness with a volume of 9.5± 1.3fl. Mature platelets stain pale blue with a granular core on a Romansky stained peripheral blood smear (Erslev & Gabuzda, 1975). They do not normally adhere to other platelets, endothelium, or other blood cells. Platelet fine structure has been the subject of several reviews (White, 1979; White, Clawson & Gerrard, 1981; White & Gerrard, 1982).

On Electron Microscopy the anucleate platelet is seen to be an extremely complex cell with systems of membranes, microtubules, microfilaments and organelles. (Fig.1.3). As a result of these findings the ultrastructure revealed by electron microscopy can be divided into four distinct regions and these are shown in Table I.

(1) The peripheral zone.

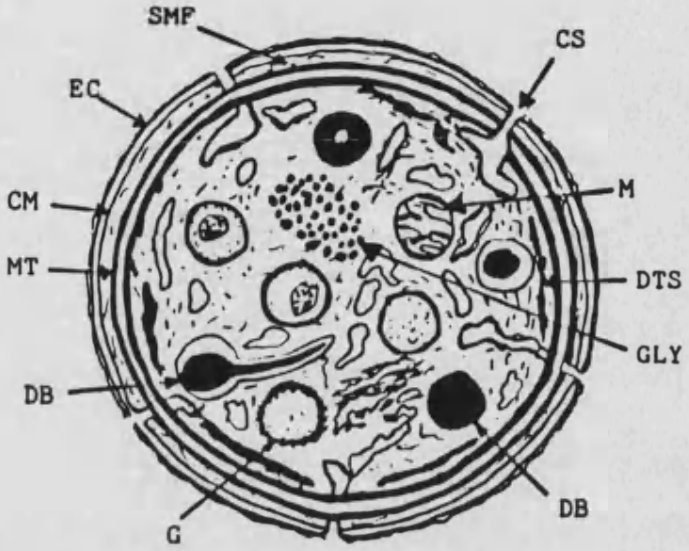
(2) Sol-gel zone.

(3) Organelle zone.

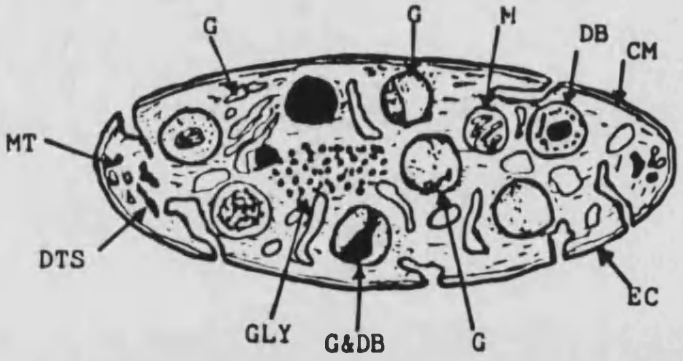
(4) The membrane system.

Zone	Structure	Function
Peripheral	Membranes	Adhesion/Aggregation
Sol-Gel	Microfilaments	Contraction
Organelle	Microtubules	Storage/Secretion
Membrane	Organelles	Channels Substances in/out

TABLE I. Ultrastructure regions of platelets.



(a) Equatorial Plane



(b) Cross Section

Fig. 1.3 Platelet Ultrastructure: (a) Equatorial Plane (b) Cross Section. Peripheral Zone: EC = Exterior Coat; CM = Trilaminar Membrane. Matrix Sol Gel Zone: SMF = Submembrane Filaments; MT = Microtubules; GLY = Glycogen. Organelle zone: M = Mitochondria; G = Alpha Granules; DB = Dense Bodies; DTS = Dense Tubular System.

1.2 The Peripheral Zone

The peripheral zone constitutes the platelet wall and invaginates extensively into the interior of the cell to form the surface connecting or open canalicular system. The peripheral zone plays an important role in maintaining platelet integrity, providing receptor sites for various stimulatory or inhibitory agents e.g. collagen, ADP, adrenaline and is responsible for platelet adhesion and providing a phospholipid surface to accelerate blood coagulation. (Fig.1.4). The peripheral zone is composed of three morphological areas, (a) exterior coat, Glycocalyx, (b) the unit membrane and (c) the submembrane region.

(a) The exterior coat is rich in glycoproteins, several of which are unique to platelets. Five major platelet glycoproteins (GPI-V) have been characterised (International Committee on Thrombosis and Haemostasis, 1981). A deficiency of certain of these glycoproteins in congenital disorders of platelet function provides evidence for their role in adhesion and aggregation (Nurden & Caen, 1976) GPI is necessary for normal adhesion and GPII & GPIII for normal aggregation. (Fig.1.4). The surface glycoproteins contain terminal sialic acid molecules contributing to a large negative surface charge which could help, by electrostatic repulsion, to prevent platelets sticking to one another or to the normal intact endothelium (Gorog, Schraufstatter & Born, 1982).

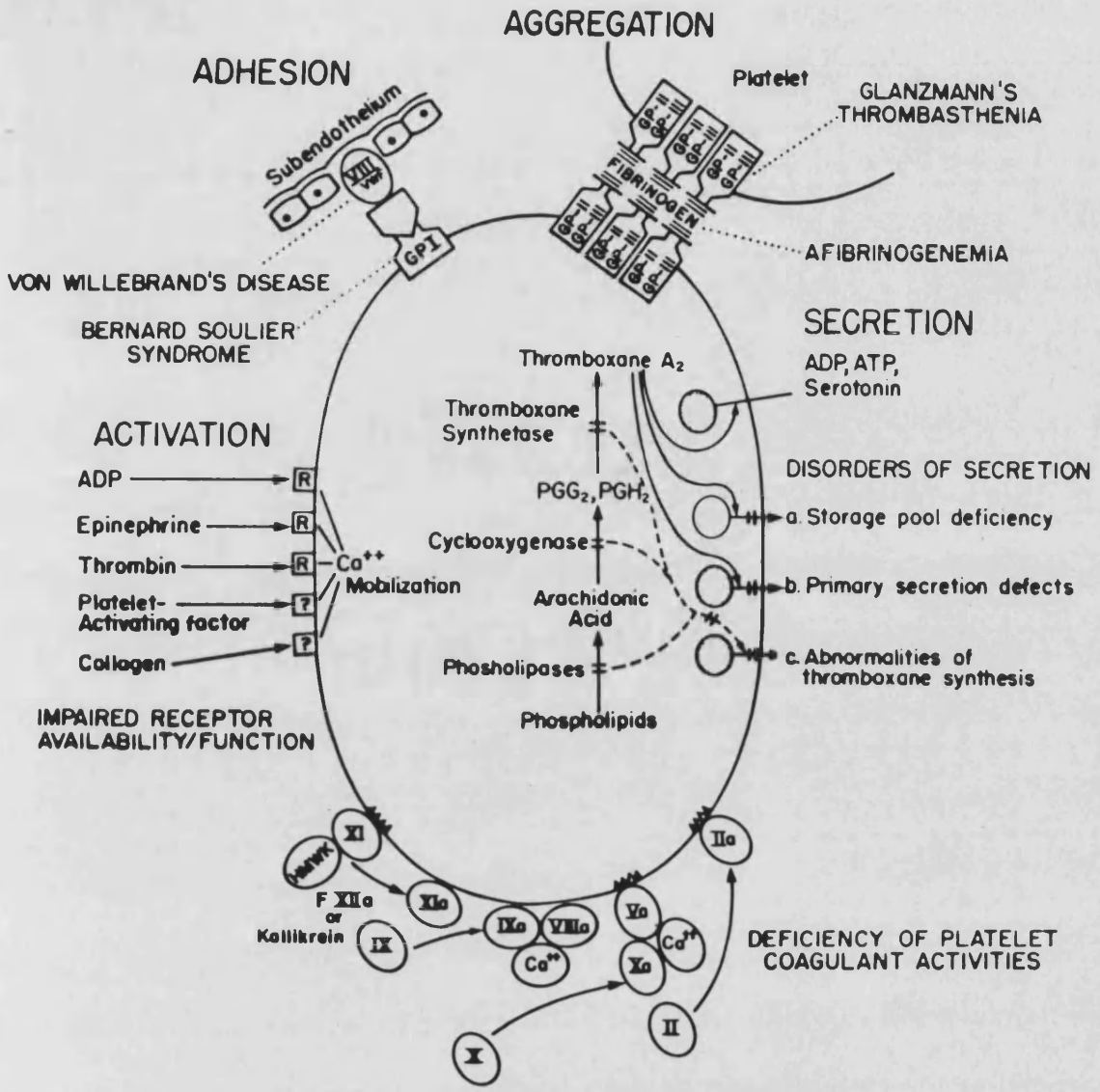


Fig. 1.4 Schematic representation of the normal platelet responses and currently recognised types of defects in platelet function. Adhesion of platelets to subendothelium is mediated by the interaction of factor VIII - von Willebrand Factor and a membrane glycoprotein (GP-I) on the platelet.

Interaction of agonists such as ADP, Adrenaline, Thrombin, Collagen and platelet activating factor with specific membrane receptors (R) results in platelet activation. This leads to mobilisation of calcium from intracellular stores. Aggregation of platelets involves glycoproteins IIb and IIIa on the platelet surface and the plasma factor Fibrinogen.

(b) The unit membrane consists mainly of a phospholipid bilayer with transmembrane protein compartments randomly dispersed. The phospholipid molecules, which are distributed asymmetrically on the two sides of the ailayer are able to diffuse laterally or become translocated from one side to the other. Important components of the platelet unit membrane include the membrane endoenzymes, the enzymes involved in membrane transport and cyclic AMP metabolism.

(c) The submembrane region constitutes the space between the unit membrane and the circumferential band of microtubules and contains a system of filamentous elements. Functionally the submembrane filaments may be involved in maintaining the platelet discoid shape, pseudopodal formation and clot retraction.

1.3 The sol-gel-zone

This zone comprises the viscous matrix inside the platelets and is composed mainly of proteins that can be assembled into fibrous elements. The microtubules are arranged in a circumferential band just beneath the cell wall in the non-activated platelet. They are composed of tubulin, polymerized into a single tubule of 25nm in diameter that is coiled to form a group of 10-24 circular profiles (Nachmias, 1980). These tubules appear to maintain discoid shape and are involved in internal contraction of platelets when stimulated.

1.4 The Organelle Zone

Several different types of organelles including mitochondria can be seen in the cytoplasm of platelets. They have a random distribution in non-activated platelets, but centralize upon activation. The main function of the organelle zone is to store important physiological substances in the granules. The electron dense granules contain calcium, adenosine-5-diphosphate, (ADP), adenosine triphosphate (ATP) and serotonin. Approximately 70% available ATP/ADP is stored in granules and is secreted during platelet aggregation. The non-dense granules are the most numerous, and cell fractions and cytochemical techniques have revealed that they can be divided into alpha granules, lysosomes and peroxisomes (Bentfeld-Barker and Bainton, 1982). Alpha granules are either Type I or II, examples of Type I (20-200 per platelet) are Beta Thromboglobulin (B-TG), Platelet Factor 4 (P.F.4), Platelet Derived Growth Factor, thrombin-sensitive protein and fibrinogen. Examples of Type II (2-10 per platelet) are lysosomal enzymes.

1.5 The Membranous System

Two important anatomical connecting systems are found in the platelet, the surface connecting system, and the dense tubular system. The former consists of a network of channels spreading throughout the cytoplasm. These channels greatly enlarge the platelet surface area and allow blood borne substances easier access to the inner parts of the platelets.

They also serve as conduits to convey substances released by the platelets. Externalization of the internalized platelet membrane appears to take place during platelet shape change (Frojmovic & Milton, 1982) possibly resulting in the exposure of functional sites.

The dense tubular system is composed of smooth endoplasmic reticulum and is very pronounced in the vicinity of the microtubules (Werner & Morgenstern, 1980). It is the major storage site for calcium which can be mobilised to regulate platelet activation, shape change and aggregation. Calcium is the pivot ion that regulates many of the cellular functions of the platelets. It is involved with initiating changes leading to adhesion, secretion and contraction. During these exchanges the resting cytoplasmic level of calcium is raised by an influx of calcium which increases cell activation via calmodulin and other proteins (Gerrard, Peterson & White, 1981). Prostaglandin metabolism also appears to be localised in this region.

Platelets have associated internal and external platelet coagulation factors. Some of these, fibrinogen and Von-Willebrand factor are necessary for normal platelet function e.g. platelet adhesion to exposed endothelial surfaces via Von-Willebrand factor, (Sakariassen, Bolhuis & Sixma, 1979; Sixma, 1981b) (Fig.1.4). Others, such as factor V on release from internal alpha granules attach to the platelet phospholipid interphases, thus enhancing prothrombin via

Factor X interaction. Specific proteins such as platelet derived growth factor play a significant role in tissue repair and healing (Ross & Vogel, 1978; Raines & Ross, 1982) by their effect on endothelial cells, smooth muscle cells and fibroblasts.

(D) Platelets and the Vascular System

1.1 The Interaction of blood platelets, vessel wall and coagulation

Whenever blood vessels are damaged there is an immediate physiological response designed to minimize and eventually arrest the loss of blood. This involves the interaction between vessel walls, circulating platelets with normal function and the coagulation plasma proteins (Fig. 1.5). In addition to the interaction of these three compartments further mechanisms are involved:- physiological, inhibitory and fibrinolytic systems which maintain a balanced equilibrium (a) localized vasoconstriction following damage, (b) platelet adhesion to injured blood vessels, (c) platelet shape change and aggregation, (d) fibrin formation via the coagulation cascade system, (e) activation of the fibrinolytic system and (f) regulation of a series of physiological inhibitors.

In this manner coagulation balances the physiological requirement of no blood loss from damaged vessels and of greater importance, no thrombosis in intact vessels.

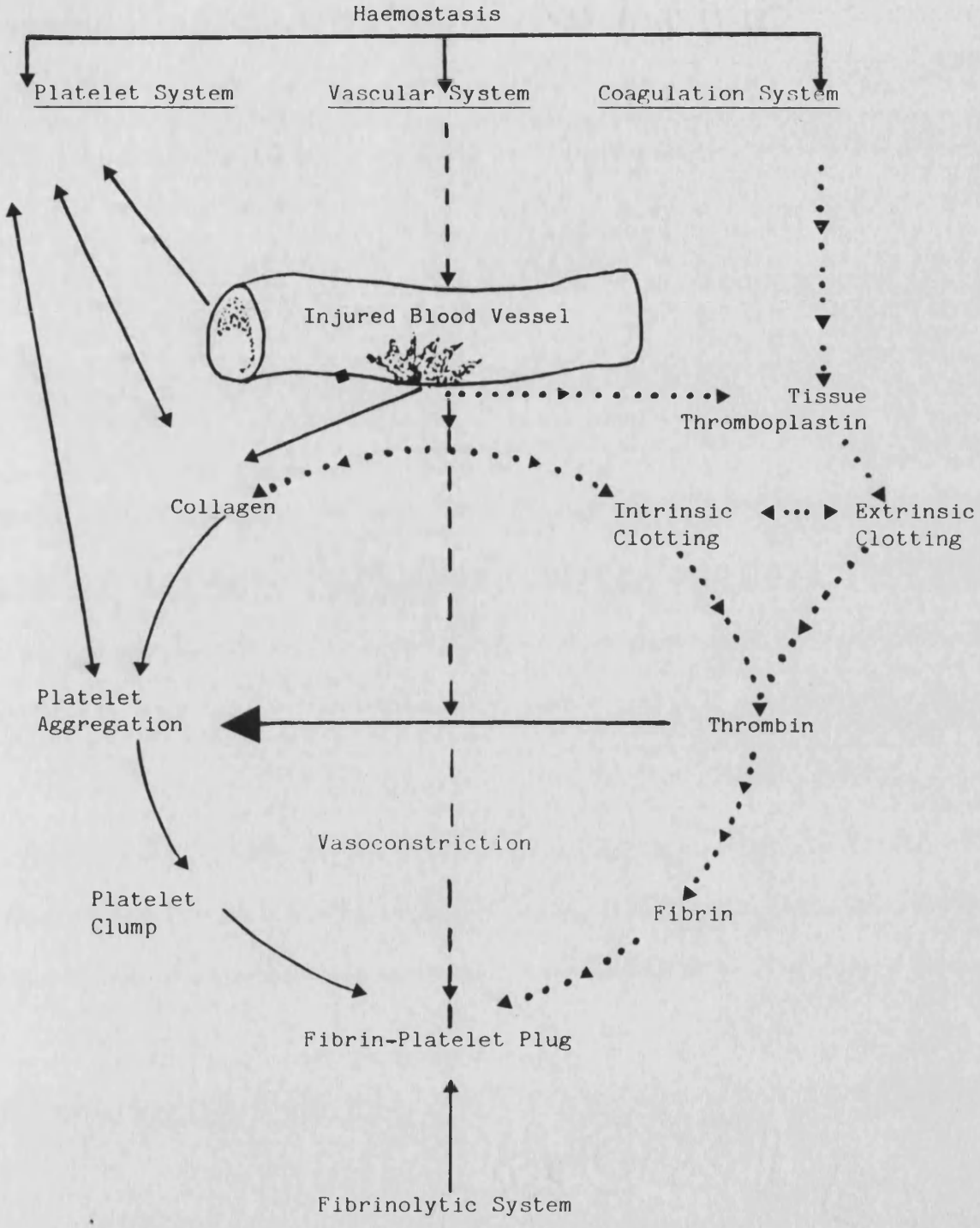


Fig. 1.5 Balanced Equilibrium between the platelet system, vascular system, coagulation system and fibrinolytic system during blood vessel damage.

1.2 Localised Vasoconstriction

Under normal physiological conditions white cells and fluids are allowed to pass through the capillary wall whilst blood and other cellular elements, in particular, platelets are retained within. In the intact microcirculation platelets circulate in close proximity to the vessel wall without adhering to each other or to the endothelium. However, following injury there is a transient vascular constriction causing turbulent blood flow, conditions which aid the deposition of platelets at the injured site. The precise mechanism that induces the vasoconstriction is not fully understood, but it is possible that the scale of response to trauma is proportional to the size and physiological site of the damaged vessel and is possibly regulated by internal pressure, exposure of subendothelium and the release of vasoactive amines from the circulating platelets.

1.3 Blood Platelets and the Vascular System

Tissue damage as seen in rheumatoid arthritis leads to the disruption of endothelial lining and permits platelets to adhere to the subendothelial tissue (Caen, 1972).
Investigations have shown that endothelial cells (a) physically monitor vessel wall integrity, (b) synthesise and store important haemostatic components, (c) produce physiological substances that inhibit platelet adherence

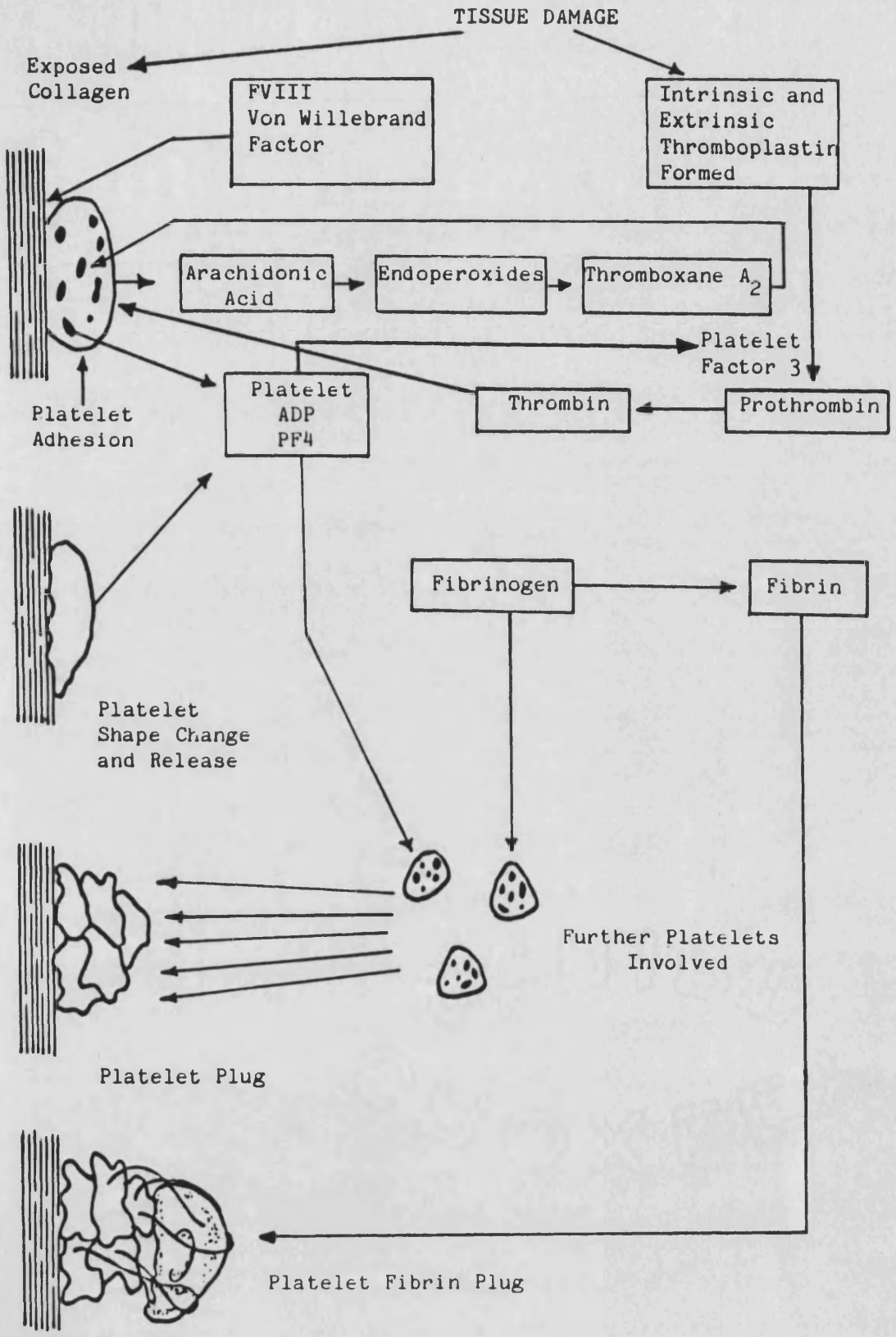


Fig. 1.6 Interaction between Platelets, Plasma Factors and Vessel Wall.

and subsequent aggregation (Caen, 1972). In a pathological state, damage to the vessel wall exposes collagen fibres. Platelets adhere to the collagen and release adenosine diphosphate and platelet degranulation occurs. This process accelerates and more and more platelets arrive, attach to the adhered platelets and platelet aggregation is initiated. Degranulation of platelets enhances further platelet aggregation and a feedback loop is maintained. These changes are accompanied by a pronounced infiltration of polymorphonuclear leucocytes, lymphocytes and monocytes thus leading to the inflammatory response.
Such events are demonstrated schematically (Fig.1.6).

1.4 Interaction between anti-aggregation activity of vessel wall prostacyclin and pro-aggregating production of platelet thromboxane A_2

The most important product of the endothelial cell is prostacyclin (PGI_2) (Moncada & Vane, 1979) an endogenous inhibitor of platelet aggregation and a very labile metabolite of arachidonic acid. It is a circulating hormone generated in venous and arterial endothelial cells from arachidonic acid via prostaglandin endoperoxides G_2 and H_2. Since the platelet has the ability to form the pro-aggregating substance thromboxane A_2 (TXA_2) from its own arachidonate pathway, there is a potential dynamic interaction between the anti-aggregatory activity of vessel

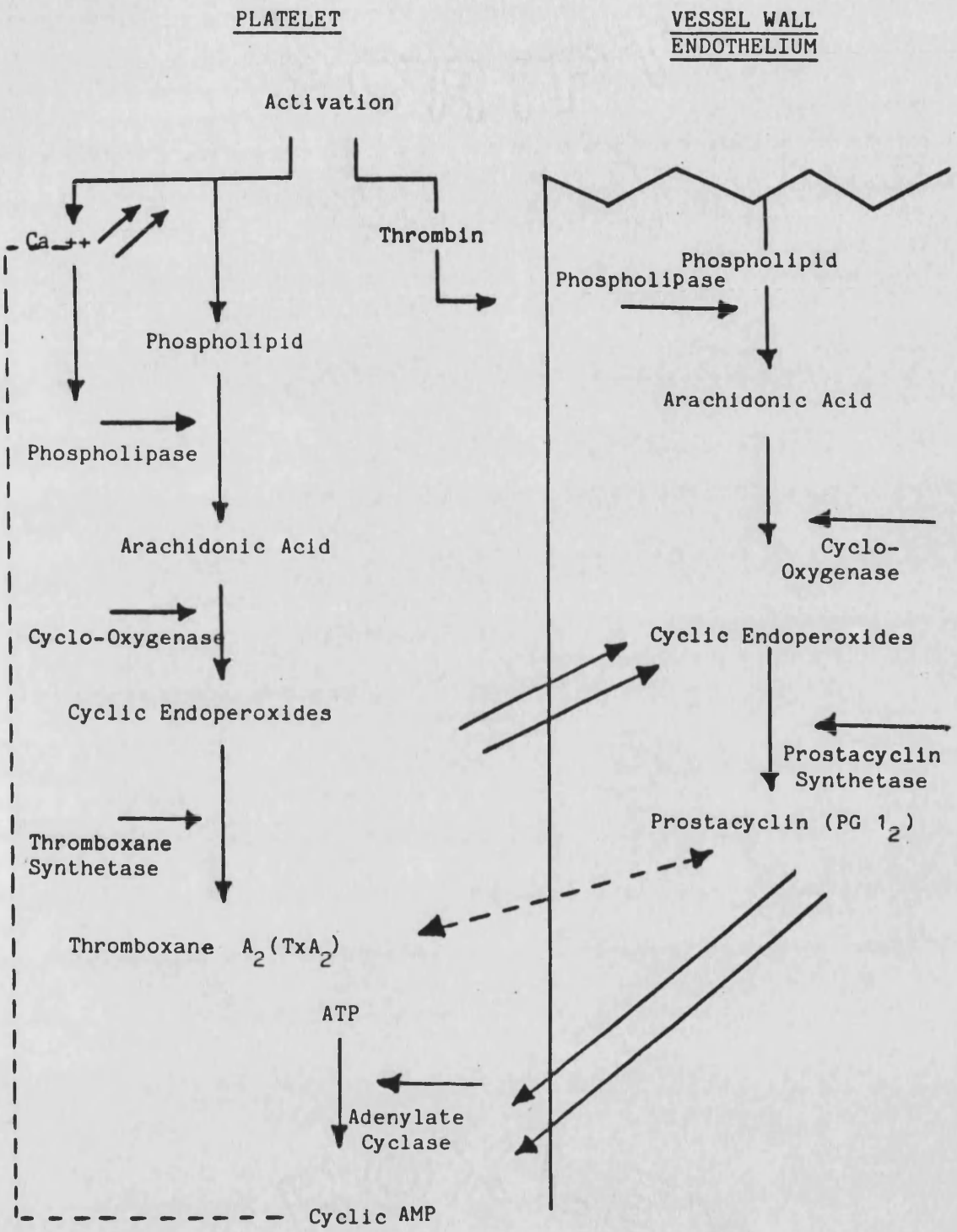


Fig. 1.7 Interaction between anti-aggregating activity of vessel wall prostacyclin and pro-aggregating production of platelet thromboxane A_2.

wall prostacyclin and the pro-aggregating production of platelet TXA_2 (Fig.1.7). There appears to be a balanced equilibrium between vessel wall PGI_2 and platelet TXA_2 which regulates platelet aggregation and therefore influences haemostasis and thrombosis.

Moncada & Vane (1979) suggested that there is continuous production and release of PGI_2 by the endothelial cells and one of the consequences of tissue damage is that not only is the subendothelial collagen exposed, but there is loss of released prostacyclin, enabling more platelet aggregates to form. This can lead to further tissue degeneration and activation of the coagulation and fibrinolytic systems.

(E) Platelet Adrenoceptors

1.1 Platelet Alpha$_2$ Adrenoceptors

In common with cells in many peripheral tissues the blood platelet carries receptors on its plasma membrane that enable this cell to respond to blood borne catecholamines, in particular to adrenaline and noradrenaline. Human platelets contain alpha-adrenergic receptors and when exposed to agonists such as adrenaline, these receptors promote platelet aggregation and secretion; enhance aggregation caused by unrelated agents e.g. ADP and thrombin (Mills & Roberts, 1967), increase calcium influx (Grant &

Scrutton, 1979; Grant & Scrutton, 1980; Owen, Feinburg & Le Breton, 1980; Owen & Le Breton, 1980) and inhibit adenylate cyclase (Jakobs, Saur & Schultz, 1976; Hsu, Knapp & Halushka, 1979). The alpha adrenoceptors of numerous tissues have been characterised according to the potency of various agonists and antagonists (Langer, 1974; Starke, Endo & Taube, 1975). These receptors have also been classified on a morphological basis as presynaptic ($alpha_2$) and postsynaptic ($alpha_1$) (Langer, 1977; Starke, 1977; Starke, 1981; Langer, 1980). Within a series of agonists, clonidine, oxymetazoline and adrenaline preferentially act presynaptically, while phenylephrine and methoxamine preferentially act postsynaptically (Starke, Endo & Taube, 1975). Within antagonists yohimbine preferentially acts on $alpha_2$ receptors and phenoxybenzamine and prazosin on $alpha_1$ receptors, (Cavero, Gomeni, Lefevre-Borg & Roach, 1980; Starke, 1981).

1.2 Adrenoceptor Stimulus - Response Coupling

$Alpha_2$ receptors can therefore be studied on platelets using selective radioligands such as (^3H) yohimbine, (Motulsky & Insel, 1982).

In a number of systems e.g. hamster adipocytes, frog skin and rat glial cells, agonist occupancy of an alpha adrenoceptor causes inhibition of adenylate cyclase and decrease in tissue cyclic AMP (Fain & Garcia-Sainz, 1980). Inhibition of

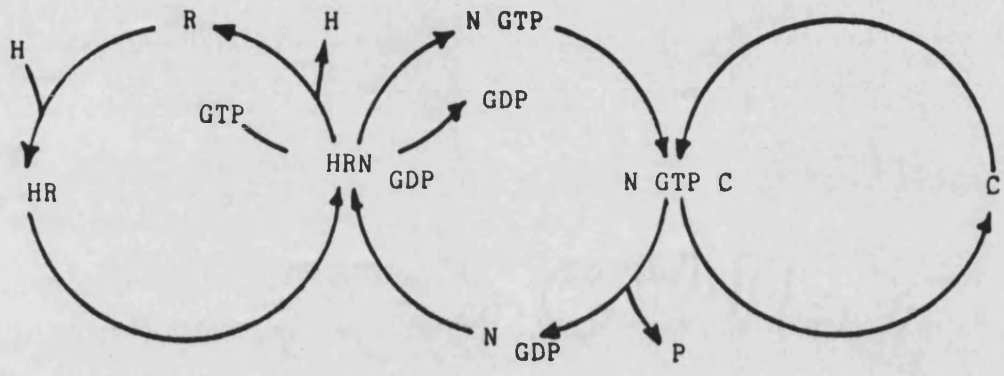


Fig. 1.8 Proposed mechanism for transmembrane coupling of Alpha adrenoceptors to adenylate cyclase. Adrenaline is designated as H, the alpha adrenoceptor as R, the nucleotide (GTP) binding protein as N, and the catalytic subunit of adenylate cyclase as C.

adenylate cyclase also results from interaction of alpha adrenergic agonists with the platelet alpha$_2$ adrenoceptor, (Lasch & Jakobs, 1979). Transmembrane coupling involving inhibition of adenylate cyclase by occupancy of an alpha adrenoceptor appears to occur by a receptor/nucleotide regulatory protein/catalytic moiety mechanism as illustrated (Fig.1.8). This is very similar to that proposed by Lefkowitz (1982) and Lefkowitz, Stadel & Caron (1983), although some observations suggest that there may be differences in the nature of the "N" proteins involved, (Nahorski, 1982). It is now well established that the presence of both extracellular divalant cations and also fibrinogen are required to observe an aggregatory response to adrenaline, (Peerschke, 1982). These requirements can be understood in the context of the exposure of fibrinogen receptors on stimulation by adrenaline, (Bennett & Villaire, 1979; Peerschke, 1982) and of the role of divalent ions in either formation of or interaction of fibrinogen with the receptor.

1.3 Responses of Platelets to Adrenaline

Several responses can result from exposure of platelets to adrenaline, depending on the species being examined. O'Brien (1963) showed a direct aggregatory response to adrenaline when added in the absence of another agonist. A proaggregatory response was noted by Mills & Roberts (1967)

in which adrenaline enhanced aggregation caused by addition of suboptimal concentrations of an excitatory agonist acting at the ADP receptor. Alternatively Yu & Latour (1977) demonstrated an inhibitory response in which adrenaline reduces the aggregatory response to a second agonist acting at a different receptor e.g. thrombin. Jakobs, Saur & Schultz (1976) found an inhibition of adenylate cyclase which in the intact cell is expressed as a prevention of the increase in cyclic AMP levels induced by an inhibitory agonist such as prostaglandin E_1.

(F) Platelet Aggregation Pathways

1.1 Platelet aggregation

Platelets respond to a number of stimuli including adenosine diphosphate (ADP) and collagen by changing shape from discoid cells to spherical spiny cells and aggregating into large clumps. Adrenaline differs from other aggregating agents in that there is no shape change and no increase in the concentration of free calcium ions within platelets (Westwick, Poll & Kakkar, 1984).

Shape change to stimuli is rapid and is associated with the exposure of previously cryptic receptors for fibrinogen, (Mustard, Kinlough-Rathbone, Packham, Perry, Harfenist & Pai, 1979; Leung & Nachman, 1982; Phillips & Baughan, 1983); and the expression of procoagulant activity which has both a

phospholipid and protein receptor component (Tracy & Mann, 1983). Finally they secrete the contents of the different storage organelles. During the late stages of the aggregation step arachidonate is liberated from certain phospholipids and rapidly converted to prostaglandins and thromboxanes which are themselves potent platelet agonists, and further activate the platelets (so called positive feedback loops, Holmsen, 1977). This response sequence results from interactions between platelet surface receptors and corresponding agonists. Since the receptors have a high degree of specificity, the sequential execution of the same responses suggest that the receptors share a common part of the signal pathway, (Holmsen, 1976; Holmsen 1978; Holmsen & Karpatkin, 1983).

Experimentally, a platelet suspension is stirred and a stimulus is added, and the coalescence of the individual platelets into aggregates is measured. The extent of aggregation depends on the concentration of the stimulating agent, the anticoagulant, the shape of stirrer bar, the rate of stirring, pH and temperature. All these parameters must be standardised to obtain reproducible results.

The three most studied pathways of autoactivation of platelets are (1) the release of ADP, (2) the liberation and metabolism of arachidonate and (3) the synthesis of Platelet Activating Factor (PAF, 1-O-alkyl-2- acetyl glycero-

phosphocholine). Each one of these pathways generates compounds which act as intercellular mediators to propagate the rapid activation of the platelets. Until recently it had been widely assumed that an increase in cytosolic Ca^{2+} concentration was the mechanism of stimulation by endogenous pathways through a common intracellular trigger, possibly calcium (Gerrard, Peterson & White, 1981; Rittenhouse, 1982). In this context, induction of aggregatory response to a weak partial agonist at the $alpha_2$ adrenoceptor by a divalent cation ionophore (Grant & Scrutton, 1980a); as well as stimulation by adrenaline of $^{45}Ca^{2+}$influx (Owen, Feinberg & Le Breton, 1980); and of intracellular Ca^{2+} mobilisation as monitored by changes in fluorescence in platelets loaded with chlortetracycline (Owen & Le Breton, 1981). This was readily accepted as an indication of a role for Ca^{2+} in the aggregatory response induced by adrenaline. However, recent observations have confused the situation and may necessitate revaluation of this postulate.

Hallam & Rink (1985) found adrenaline induces no detectable increase in cytosolic Ca^{2+} concentration in platelets treated with aspirin and loaded with the intracellular Ca^{2+} indicator Quin II. $^{45}Ca^{2+}$ influx induced by adrenaline can only be detected in association with a secretory response when these two parameters are evaluated in the same sample (Clare & Scrutton, 1983b; Clare & Scrutton, 1984). Using a different protocol, Brass & Shattil (1982) and Brass & Shattil (1984) also failed to show stimulation of net Ca^{2+}

uptake by adrenaline although both these agonists and ADP increased Ca^{2+} redistribution across the plasma membrane.

1.2 ADP Pathway

The addition of exogenous ADP to platelet rich plasma stimulates shape change and aggregation. In washed platelet suspensions, when physiological concentrations of calcium and magnesium ions are present, this primary aggregation is followed by spontaneous disaggregation with no release of dense granules. In platelet suspensions with Ca^{2+} concentrations of 30-50μM, such as citrated platelet rich plasma and within a narrow range of concentration of ADP, a second stage of aggregation follows the primary aggregation and a biphasic aggregation curve is recorded. The liberation of arachidonic acid and its subsequent metabolism as well as the release of granules are associated with the second burst of aggregation. The ability of acetylsalicylic acid and scavengers of released ADP to inhibit this second phase of aggregation suggests that both the release of ADP and arachidonate metabolites are involved (Packham, Guccione, Chang & Mustard, 1973).

There are at least two hypotheses on the nature of the receptor for ADP and the mechanism of transmission of the stimulatory signal into the cell. The first is the binding of ADP to a nucleotide diphosphokinase (NDK) on the platelet

surface. This is consistent with the conversion of exogenous ADP to ATP during platelet aggregation (Mustard, Packham, Perry, Guccione & Kinlough-Rathbone, 1975).

Mechanistically, the shunting of endogenous ATP to act as phosphate and energy donor to this process and away from normal phospholipid synthesis or cyclic adenosine monophosphate (cAMP) production, could trigger the cellular response (Lam, Guccione, Packham & Mustard, 1982). Within the inositol phospholipids, a decreased synthesis of triphosphatidylinositol upon stimulation has been reported (Lapetina, 1982). However, photocoupling of an ADP analogue to the receptor for ADP has located a protein that is different from the NDK enzyme, suggesting that indirect effects on the NDK, or perhaps a molecular complex may be operative (MacFarlane, Mills & Scrivastava, 1982).

A second hypothesis is the reduction of cAMP levels within the platelet by the binding of ADP to a regulatory portion of the adenylate cyclase complex (MacFarlane, Mills & Srivastava, 1982). The cAMP levels in platelets drop slightly upon stimulation, and large increases of cAMP inhibit platelet stimulation by powerful stimuli. However it is not clear how decreases in cAMP would initiate the intracellular events that occur prior to aggregation (Haslam, 1973; Haslam, 1975).

1.3 Arachidonic Acid Pathway

The second pathway of platelet stimulation is the release of arachidonic acid from membrane phospholipids by the direct action of phospholipase A_2 or by the indirect action of phospholipase C, diglyceride lipase and monoglyceride lipase (Prescott & Majerus, 1983). The intracellular location of the phospholipid pool that donates arachidonate and the species of phospholipid remain controversial.

The arachidonic acid is metabolised via the cyclooxygenase pathway with the formation of prostaglandins (PG) G_2 H_2 and E_2, thromboxane A_2 (TXA_2), and malondialdehyde, (MDA) or via the 12-lipoxygenase pathway with the formation of 12-L-hydroxy-5,8,10-heptadecatrienoic acid (12-HETE) (Marcus, 1978). Platelet stimulation by thrombin, calcium ionophore, collagen and ADP or adrenaline in low calcium conditions, is associated with activation of membrane phospholipases. Platelets can also metabolize exogenous arachidonate to produce PGH_2 and TXA_2, both of which are stimulants of platelet aggregation (Marcus, 1978). Platelets can also produce PGE_2, which inhibits platelet aggregation by raising intracellular cAMP concentrations. There is a range of arachidonate concentrations (100 - 300μM) that inhibit platelet aggregation, while both lower and higher values have no apparent effect. The reason for this anomaly is unknown.

One important consideration when inhibitors of one pathway of arachidonate metabolism are used is that metabolites from the other pathway will be increased. Thus the inhibition of cyclooxygenase by acetylsalicylic acid causes an elevation in 12-HETE products (Marcus, 1978).

1.4 Ether Phospholipid Pathway

A third pathway of platelet activation has been suggested to be the formation of Platelet Activating Factor (PAF), although the site of synthesis and catabolism and mechanism of stimulation within platelets remains unknown. Other blood cells such as macrophages, neutrophils, basophils and monocytes produce PAF (Pinckard, McManus & Hanahan, 1982). The chemical structure of PAF has been determined to be 1-O-alkyl-2-acetyl-sn-glycerophosphorylcholine (Demopoulos, Pinckard & Hanahan, 1979) and is therefore related to other phospholipids. Phospholipids in human platelets consist of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin (Cohen & Derksen, 1969; Owen, Hutton, Day, Bruckdorfer & McIntyre, 1981; Mahadevappa & Holub, 1982). The synthetic pathways of PAF are emerging, one suggestion is that the action of phospholipase A_2 forms 1-O-alkyl-2 phosphatidylcholine (lyso-PAF) with subsequent acetylation to form PAF (Albert & Snyder, 1983; Albert & Snyder, 1984). The activation of

platelets by PAF has been suggested to be through a receptor binding and initiation of the phosphotidylinositol cycle (Lapetina, 1982; Billah & Lapetina, 1982a; Billah & Lapetina, 1983); or the inhibition of adenylate cyclase (Haslam & Vanderwel, 1982). Platelet activation by thrombin, collagen, ADP and adrenaline have all been shown to activate the phosphatidylinositol cycle similarly (Rittenhouse-Simmons & Deykin, 1981; Leung, Vickers, Kinlough-Rathbone, Reimers & Mustard, 1983b). Once the synthesis and catabolism of PAF and the effects of various inhibitors are classified, a fourth pathway of platelet activation may well emerge.

1.5 Synergisms

Platelet stimuli when used together, result in stronger aggregation responses than those observed independently. This synergistic phenomenon, while not understood has been repeatedly observed for almost all pairs of activating stimuli (O'Brien, 1964a; Mills & Roberts, 1967; Grant & Scrutton, 1980b; Huang & Detwiler, 1981; Cameron & Ardlie, 1982). An important distinction must be made between synergistic actions that yield enhanced primary aggregation and eventual secondary aggregation and release of granules. Huang & Detwiler (1981) stimulated platelets with two stimuli such as ADP and high concentrations of thrombin. They found enhanced primary aggregation but no secondary aggregation or release of granules even when 30μM ADP was employed as long as

the thrombin concentrations remained fairly low. Increasing thrombin levels, at low ADP concentrations induced secondary aggregation. A closely related situation has been reported in which platelets become refractory to a second stimulation by the same agent after disaggregation (Rao & White, 1981). However, if sub-activating concentrations of adrenaline are added before the second stimulation by the same stimulus, reaggregation will result (Rao, Reddy & White, 1981). Clearly more than just simple convergence of excitation pathways to a common intracellular event is in operation.

1.6 Beta-Thromboglobulin (B-TG)

When blood platelets aggregate several proteins and other factors are liberated. One of the platelet proteins released is B-thromboglobulin (B-TG) (Moore, Pepper & Cash, 1975; Bolton, Ludlam, Moore, Pepper & Cash, 1976; Moore & Pepper 1976). This protein has been purified and characterized, (Begg, Pepper, Chesterman & Morgan, 1978) although its function is, as yet, not perfectly clear. The protein has a molecular weight of 36,000 and is thought to comprise six identical subunits. It is probably located in the alpha granules of the blood platelets (Moore, Pepper & Cash, 1975). It is the most abundant platelet specific protein and is possibly a "granule packing protein" stablising the active constituents in the alpha granules (Moore & Pepper, 1976). B-TG is only present in minute amounts in other tissues and can therefore be considered to be a platelet specific

protein, the release of which is a clear marker of platelet release and aggregation (Zahavi & Kakkar, 1980).
When B-TG is released from platelets its retention time in plasma is relatively short, with a half life of around 100 minutes (Dawes, Smith & Pepper, 1978). Therefore, a measurement of its concentration in platelet poor plasma represents a dynamic equilibrium between release and clearance. If the phenomenon responsible for its release is only transitory, as in formation of thrombi in deep vein thrombosis or myocardial infarction, then concentrations may rise only transiently, (Pepper & Ludlam, 1977; Denham, Fisher, James & Hassan, 1977). However, a more continuous lesion such as vasculitis or chronic inflammation may well give rise to a "steady state", thus reflecting constantly elevated rates of platelet aggregation or destruction.

(G) Platelets and Inflammation

1.1 Immune Complexes

The contribution of platelets to the inflammatory process associated with immunological injury has been suggested by several workers (Pfueller & Luscher, 1974; Nachman, 1978). Although human platelets participate in the inflammatory process, little attention has been paid to their possible involvement in the pathogenesis of rheumatoid arthritis (RA) (Colli, Maderna, Tremoli, Colombo & Canesi, 1982). Previous studies have described an increase in platelet count in a large percentage of rheumatoid patients, as well as an

apparent relationship between thrombocytosis and several biological and clinical markers of active disease (Bean, 1965; Selroos, 1972; Hernandez, Rowan, Kennedy & Buchanan, 1975; Hryszko, Pietruska, Bernacka & Bogdanikowa, 1975).

Several aggregating agents e.g. collagen, thrombin, ADP, and adrenaline may induce aggregation and the release of platelet contents from storage granules (Holmsen, Salganicoff & Fukami, 1977). Also, aggregated IgG and immune complexes, unrelated to platelet antigens or other platelet constituents can induce aggregation and release of platelet contents (Pfueller & Luscher, 1972; Pfueller & Luscher, 1974; Luscher & Pfueller, 1978). Immune complexes may also be phagocytosed by platelets (Zimmerman & Kolb, 1976).
Depending on the nature of the inducer, the substances which may be released or secreted from platelets include ADP, 5-hydroxytryptamine 5HT, calcium, acid hydrolases and other mediators of inflammation.

Human platelets have receptors for the Fc region of aggregated IgG (Kulka, Bocking, Ropes, & Bayer, 1955; Weksler & Coupal, 1973), and the interaction with IgG/immune complexes appears to be a direct binding of the Fc region of IgG to the platelet Fc receptors.
There is also evidence of receptors for complement factor C3 on human platelets (Zimmerman & Kolb, 1976) thus (Ab-Ag) complexes may induce platelet activation both directly and

via the complement system resulting in platelet aggregation and release, followed by toxic events on endothelial cells (Henson & Cochrane, 1969a; Henson & Cochrane, 1969b; Henson, 1970; Jorgensen, Hovig, Rowsell & Mustard, 1970; Israels, Nisli, Paraskevas & Israels, 1973). Furthermore, platelets are capable of stimulating chemotaxis via complement activation in a manner similar to leucocytes (Weksler & Coupal, 1973) and therefore may participate in the early stages of inflammation in rheumatoid arthritis or any other connective tissue disease. Thus human platelets are both haemostatic and inflammatory cells which can be activated by immunological and inducer mechanisms. Platelet activation could therefore play a very important role in the pathogenesis of rheumatoid arthritis (Fig.1.9).

The contribution of platelets to the inflammatory process, linked to immunological injury, may lead to the formation of microthrombi and the exposure of platelets to insoluble antibody-antigen (Ab-Ag)complexes, and subsequent release of biologically active components and lysosomal enzymes, which in turn increase vascular permeability. (Nachman & Ferris, 1968; Henson & Cochrane, 1969a; Henson & Cochrane, 1969b; Henson, 1970; Chesney, Harper & Colman, 1974).

1.2 Thrombocytosis

Hutchinson, Davis & Jayson (1976) suggest that thrombocytosis accompanies rheumatoid arthritis and is due

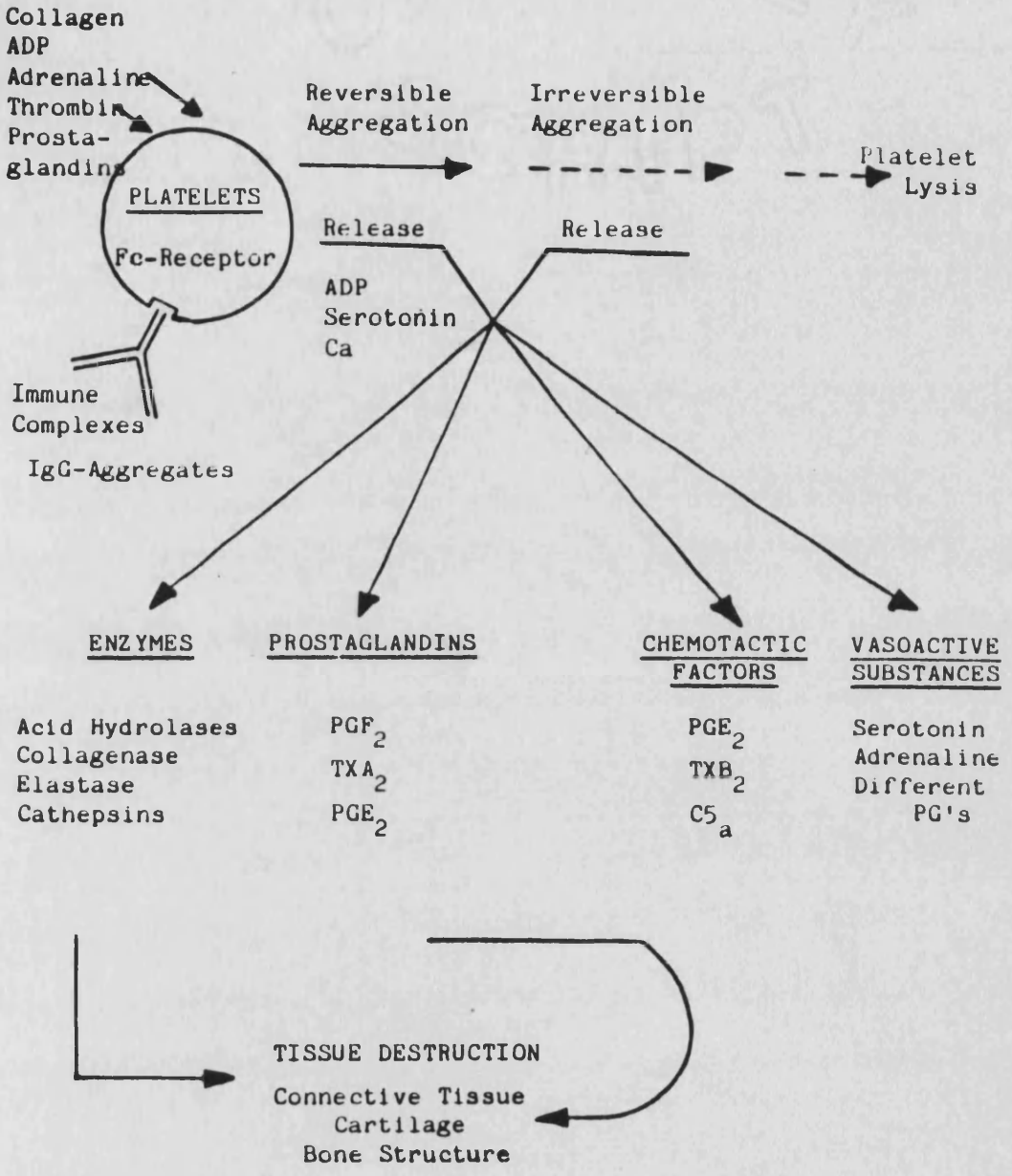


Fig. 1.9 A proposed model of the possible roles of platelets in the pathogenesis of synovitis in Rheumatoid Arthritis

to a compensatory increase in platelet production associated with active intravascular coagulation. Platelet thrombi, together with obliterative microangiopathy and platelet aggregation on altered endothelial surfaces, have been demonstrated in histological studies of active lesions in synovial tissue obtained from patients with early and chronic rheumatoid arthritis (Schumacher, 1975).

Fink, Piening, Fricke & Deicher (1979) found that the addition of sera from patients with sero-positive classical rheumatoid arthritis to platelet rich plasma taken from normal patients will also induce platelet aggregation. Several other studies implying that the reactivity of blood platelets is altered by immune complexes in some types of arthritis have been suggested. (Pfueller & Luscher, 1972; Weksler & Coupal, 1973; Luscher & Pfueller, 1978).

Biochemical analysis of platelets obtained from patients with rheumatoid arthritis has shown increased content of acid mucopolysaccharide together with low 5HT levels, a diminished protein content, a possible decrease in acid polysaccharide activity and reduced amounts of connective tissue activating peptide III (Smith & Castor, 1978). Along with increased platelet counts the turnover of plasma fibrinogen was found to be elevated in patients with rheumatoid arthritis as well as increases in fibrin degradation products (Barnhart, Riddle & Bluhm, 1967; Bennett, Eddie-Quartey & Holt, 1972; Selroos, & Wegelius,

1973b; Selroos, Petterson & Wegelius, 1973c; Hutchinson, Davis & Jayson, 1976).

Thus the presence of an over-compensated intravascular coagulation and fibrinolytic system may make a significant contribution to the inflammatory process in patients with rheumatoid arthritis, and may be brought about by over-active platelet aggregation in the synovial fluid (Endresen, 1981).

In the authors view, the likely systems for the action of penicillamine must have exaggerated activity in the inflammatory process and be influenced in vitro at drug levels achieved in vivo.

Many of the cellular and subcellular mechanisms of the blood system have been examined in rheumatoid arthritis, however, one of the cellular aspects that requires intensive studies is the blood platelet. This cellular consituent of blood was chosen, since, as discussed, is probably intimately involved in the acute inflammatory reaction found in rheumatoid arthritis.

The aim of this thesis was (1) to determine the possible role and significance of increased platelets in rheumatoid arthritis, and (2) to identify the effects of D-pen especially on the aggregating responses of platelets.

CHAPTER TWO:

MATERIALS AND METHODS

MATERIALS

2.1 Materials	Suppliers
All chemicals except where otherwise stated:	Sigma Chemical Co. Ltd., & British Drug Houses Ltd. Poole.
4ml Labco blood tubes (B.S.4851) Final concentration of 1.5mg/ml of EDTA Potassium Ethylene-Diamine-Tetra-Acetic Acid:	Labco Ltd., Marlow, Bucks.
Sterilin capped 20ml polystyrene universal containers:	Sterilin Ltd., Feltham, England.
Plastic transfer pipettes:	Alpha Laboratories, Eastleigh, Hants.
60ml plastic syringes + 19g Sabre hypodermic needles:	Gillette UK Ltd., Isleworth, Middlesex.
Precision micropipettes:	B.C.L., Boehringer Mannheim House, Lewis, East Sussex.

Coulter reagents: Blood diluent Isoton III Lysing agent Lyse S III Detergent Isoterge III (1.2 dilution with Isoton III) Bleaching agent. Hypochlorite (2%V/V solutions)	Coulter Electronics Ltd., Northwell Drive, Luton, Beds.
4c Plus blood control:	
Scintillation vials: & plastic tubes. 75x12mm	Richardsons of Leicester, Leicester.
R.I.A. B.-Thromboglobulin: (2μCi ^{125}I-labelled B-TG)	Radiochemical Centre Ltd., Amersham, Bucks.
Insta-Gel scintillation fluid:	Packard Instrument Ltd., Caversham, Berks.
[O-methyl-^{3}H] yohimbine: Specific activity: 72 curies/mmol, 200 mCi/mg, Radioactive concentration 0.9mCi/ml:	Radiochemical Centre Ltd., Amersham, Bucks.
Distamine: (D-penicillamine)	Dista Products Ltd., Basingstoke, Hampshire.

Myocrisin:	May & Baker Ltd.,
(sodium aurothiomalate)	Dagenham, Essex.

2.2 Equipment	Suppliers
Coulter S Plus III	Coulter Electronics Ltd.,
+ Data Terminal:	Northwell Drive, Luton,
	Beds.
Platelet Aggregometer AT8:	Accutech Ltd.,
	Littleborough, Lancs.
I.E.C. Centra 4 X centrifuge:	Damon/I.E.C. (UK Ltd).,
I.E.C. Centra 7R refrigerated	Chestnut House,
centrifuge:	North Dunstable, Beds.
L.K.B. Flat Bed	L.K.B. Biochrom Ltd.,
Chart Recorder:	Science Park,
L.K.B. Ultrospec 4050	Cambridge.
U.V. Spectrophotometer:	
L.K.B. 1260 Multigamma	
II Counter:	
Grant water bath:	Grant Instruments
Grant boiling water bath:	(Cambridge) Ltd.,
	Barrington,
	Cambridge.

Gallenkamp vortex mixer:	Gallenkamp, (Fisons PLC Scientific Division), Loughborough, Leics.
Tri-Carb 400 series Liquid scintillation counter:	Packard Instrument Ltd., Caversham, Berks.

2.3 Routine buffers and reagents

2.3.1 Phosphate buffered saline (PBS) x 10

	g/litre
Sodium chloride	80.00
Potassium chloride	2.00
Disodium hydrogen phosphate	11.50
Potassium dihydrogen phosphate	2.00 pH 7.3

The above were dissolved in 1 litre of distilled water, filter sterilised (millipore filter 0.45 um pore size) and stored at 4°C until required.

Before use, the solution was diluted to ten times its volume with distilled water. To this was added the equivalent of:

0.1 g/litre calcium chloride

0.1 g/litre magnesium chloride

The pH was adjusted to 7.3 with 1M sodium hydroxide solution.

2.3.2 Tyrodes buffer

	g/litre
Sodium chloride	160.00
Potassium chloride	4.00
Sodium bicarbonate	20.00
Sodium dihydrogen phosphate	1.00

The above were dissolved in 1 litre of distilled water, filter sterilised (millipore filter 0.45 μm pore size) and stored at $4^{o}C$ until required.

Before use the solution was diluted to twenty times its volume with distilled water. The pH was adjusted to 7.35 with 0.1N hydrochloric acid.

2.3.3 Thiobarbiturate reagent

2 - Thiobarbituric acid	40.0g
5N Sodium hydroxide	250ml

The pH was adjusted to pH 12.0 with 70% perchloric acid.

Distilled water	250ml
final volume	500ml

and stored at $4^{o}C$ until required. Fifteen minutes before use, 20ml of the above reagent was mixed with 10ml of 7% perchloric acid.

2.3.4 Anticoagulants

3.2% Sodium citrate anticoagulant (buffered) was used for studies on platelet function in the proportion of 1 vol of citrate to nine volumes of blood.

	g/litre
Tri sodium citrate dihydrate	32.00
N-2-hydroxyethyl-piperazine-N-2-ethane sulphonic acid (HEPES)	50.00

The above were dissolved in 1 litre of distilled water, filter sterilised (millipore filter 0.45μm pore size) and stored at 4°C until required.

This maintains the pH of the anticoagulated plasma at 7.4 the optimal pH for platelet aggregation studies.
The storage of platelet rich plasma results in loss of dissolved carbon dioxide and a rise in pH, the use of HEPES and capped plastic tubes prevents this.

2.4 Platelet aggregation reagents

2.4.1 ADP:- Adenosine-5-diphosphate

The stock solution of ADP 1mM/l was stored at -20°C in 0.5ml aliquots. Working solutions were prepared by diluting the

stock solution with 0.15M NaCl to give appropriate final concentrations. One volume of aggregating agent was added to 10 volumes of platelet rich plasma. All dilutions were made in 0.15M NaCl immediately before use and kept on ice during experiments.

2.4.2 Adrenaline:- Epinephrine bitartrate

The stock solution of adrenaline 1mg/ml was stored at 4^0C. Working solutions were prepared by diluting the above stock solution with 0.15M NaCl to give appropriate final concentrations. One volume of aggregating agent was added to 10 volumes of platelet rich plasma. All dilutions were made in 0.15M NaCl immediately before use, kept on ice and protected from sunlight.

2.4.3 Collagen:- (calf skin - Type 1)

The stock solution of collagen 1mg/ml was stored at 4^0C and working solutions were made in Tyrodes buffer to the desired concentrations. One volume of aggregating agent was added to 10 volumes of platelet rich plasma. All appropriate dilutions were made in Tyrodes buffer immediately before use and kept on ice during experiments.

"Final concentration" refers to concentration of reagent after the addition of platelet rich plasma.

METHODS

(A) Measurements of haemoglobin and platelet parameters

Haemoglobin:- g/dl, platelet count:- x 10^9/l, platelet crit:- (Pct)% and mean platelet volume (MPV):- femtolitres were measured on the coulter model S-Plus III using a fresh homogenised E.D.T.A. sample of whole blood and examined within two hours of venepuncture.

2.1 Haemoglobin concentration

This was measured photometrically as cyanmethaemoglobin after exposure to a combined lysing haemoglobin conversion reagent for at least twenty three seconds. All tests were performed in triplicate and the mean value recorded.

2.2 Platelet count

The model S-Plus III separates platelets by differential gating, measured by electrical impedance and uses the log normal principle to derive all the platelet parameters. Particles of 2-20 femtolitres are channelised from the red blood cell / platelet line and a histogram generated. The smoothed distribution curve is then scrutinised between 3-15 femtolitres for the mode.

2.3 Mean platelet volume

The model S-Plus III in addition to the platelet count, generates the platelet crit (Pct) and the mean platelet volume (M.P.V.)

The M.P.V. is calculated from the following formula:

$$M.P.V. = \frac{\exp(\mu + \delta^2)}{2}$$

where u is the log median and δ the log standard deviation.

2.4 Platelet crit

The platelet crit is the product of the platelet count and the mean platelet volume. Platelet crit correlates closely with the platelet count, which indicates that the number of platelets is the major determinant. There is an inverse relationship between platelet size and count which leads to a constant Pct.

The (Pct) is calculated from the following formula:-

$$Pct = \frac{\text{platelet count} \times M.P.V. \%}{10000}$$

(B) Determination of platelet aggregation

2.1 Preparation of platelet rich samples from controls and various patient groups

40ml of venous blood was taken from an antecubital vein by clean venepuncture and with minimum stasis. The blood was mixed with an anticoagulant 3.2% sodium citrate (HEPES) in the proportion of one volume of citrate to nine volumes of blood.

Platelet rich plasma (PRP) and platelet poor plasma (PPP) was prepared by centrifugation at room temperature at 120g for fifteen minutes and 2,000g respectively for twenty minutes. Platelet suspensions were prepared by removing PRP with plastic transfer pipettes to a second plastic universal container. The platelet rich plasma was then counted in a model S Plus III Coulter Counter and diluted if necessary with autologous PPP to give an average platelet count containing $200\text{-}250 \times 10^9/l$. All plastic containers of platelet rich plasma were capped immediately after counting to maintain pH and to minimise any loss of carbon dioxide. All measurements were performed within three hours of blood collection.

2.2 Platelet aggregation

Platelet aggregation was measured in vitro according to the turbidometric method of Born (1962). The measurements were

performed in a single channel aggregometer (Accutech) utilising 1ml of platelet rich plasma and 100µl of the standard aggregating agents, ADP, adrenaline and collagen. Platelet rich plasma was placed in a plastic cuvette warmed to 37°C in the heating block of the instrument and constantly stirred at 900 r.p.m. by use of a siliconised magnetic stir bar. Light transmission was monitored continuously on a chart recorder. The addition of the aggregating agent results in the formation of increasingly larger platelet aggregates with a corresponding decrease in optical density. The change in optical density was recorded as a tracing by the chart recorder for all the various concentrations of agonists, controls, and patient groups, and was standardised for each sample.

The transmission was adjusted to 0% for platelet rich plasma and to 100% with the corresponding platelet poor plasma. The platelet rich plasma was incubated at 37°C for exactly two minutes before the various concentrations of ADP, adrenaline and collagen were added. Maximum time for aggregation was five minutes and all traces were plotted on a chart recorder as a percentage transmission.

Dose response curves to increasing concentrations of ADP, adrenaline, and collagen were obtained in all groups and the concentration inducing 50% aggregation was calculated and used as an index of aggregability. E.C.50: the estimated concentration at which primary aggregation occurs at half its maximum velocity.

(C) Determination of beta-thromboglobulin

2.1 Preparation of plasma samples from controls and rheumatoid groups

2.5ml of venous blood was taken from an antecubital vein by clean venepuncture and with minimum stasis. The blood was immediately transferred to a cooled plastic tube containing powdered E.D.T.A. 2.75mg/ml and Theophylline 0.15mg/ml. These tubes were stoppered, mixed gently and cooled in crushed ice for 30 minutes. The samples were then centrifuged at 4°C at 3,000g for 30 minutes.
All sample tubes were performed in duplicate.

Centrifugation must be carried out within three hours of blood collection otherwise platelets may disrupt releasing their contents and giving false high levels of Beta-thromboglobulin. After centrifugation the top 500µl was carefully removed and transferred to a second plastic tube.

2.2 Standards

The aluminium closure foil and rubber stopper was carefully removed from the Beta-thromboglobulin human reference standards and 500µl of distilled water added with a precision micropipette.

The stopper was then replaced and the contents of each vial mixed by gentle swirling and inversion until the contents had dissolved completely and a homogenous solution obtained.

Standards contained 10ng/ml, 20ng/ml, 50ng/ml, 100ng/ml, and 225ng/ml Beta-thromboglobulin.

2.3 Antiserum

10ml of distilled water was added to 1 vial containing anti Beta-thromboglobulin and mixed by gentle inversion.

2.4 ^{125}I labelled Beta-thromboglobulin

10ml of distilled water was added to 1 vial containing 2µCi ^{125}I labelled Beta-thromboglobulin, and again mixed by gentle inversion.

2.5 Total Beta-thromboglobulin Assay (B-TG)

The B-TG was measured by radioimmunoassay using Amersham Beta-thromboglobulin reagents according to the method of Ludlam, Moore, Bolton, Pepper & Cash, (1975).

Assay Procedure was as follows:-

Standard tubes containing	**Test tubes containing**
10, 20,50, 100, 225ng/ml	
50µl of above standards	50µl of unknown plasma
200µl ^{125}I labelled B-TG	200µl ^{125}I labelled B-TG
200µl of anti B-TG serum	200µl of anti B-TG serum

The above sets of tubes were vortex mixed, and incubated for 1 hour at room temperature. After incubation and the addition of 500µl ammonium sulphate solution to standards and tests, all tubes were again vortex- mixed, and then centrifuged for 15 minutes at 1,500g at 15°C

All supernatant fluid was decanted and allowed to drain inverted for 5 minutes. After draining the radioactivity in the precipitates was counted in a LKB 1260 Multigamma II Counter.

Based on this method a calibration curve for the total Beta-thromboglobulin was determined using the known standards and calculating the means of the duplicate standards. The total Beta-thomboglobulin was determined for the various patient groups and control group by reading directly from the calibration curve and recorded as ng/ml Beta-thromboglobulin.

(D) Determination of malonyldialdehyde

2.1 Preparation of platelet samples from controls and rheumatoid groups

15ml of venous blood was collected into 3.2% sodium citrate and centrifuged at 150g for 15 minutes at 20°C to obtain platelet rich plasma (PRP) and a platelet count performed on the coulter S Plus III. Samples of PRP (0.9ml) were transferred to four tubes and the platelets sedimented by further centrifugation at 1,500g for 15 mins at 20°C.

The supernatant platelet poor plasma (PPP) was decanted off and the platelet count performed again and recorded. The tubes were then inverted to drain for 2 minutes to remove all plasma, and the platelet buttons were resuspended in 0.9ml of phosphate buffered saline (PBS).

2.2 Assay of malonyldialdehyde

For each normal control or patient, two platelet samples (0.9ml) were treated with 100µl of PBS as blank, and two samples with 100µl of one of the stimulating reagents, adrenaline, collagen and ADP. The suspensions were then incubated for 60mins at 37°C in a waterbath.
After incubation, 1ml of the 2-thiobarbituric acid solution was added to each tube which was vortex mixed to terminate the

reaction, and after capping, the tubes were transferred to a boiling waterbath for 10 minutes. The tubes were then removed, cooled on ice, and centrifuged at 2,000g for 15 minutes at 4°C. The supernatants were then decanted and absorbance measured at 532nm against the appropriate blank in an L.K.B. ultrospec U.V. spectrophotometer.

2.3 Calculation

The number of n moles of malonyldialdehyde / 10^9 platelets were then calculated by the following formula:-

$$\frac{A.Vf.}{N.Vi.E} \times 10^9$$

Where A = optical density of samples at 532nm

Vf = final volume of sample (1ml)

Vi = initial volume of sample (0.9ml)

N = number of platelets in sample (platelet count of PRP-platelet count of PPP)

E = Molar extinction coefficient of malonyldialdehyde (1.55×10^5)n moles

The results were expressed as mean ± SD of the duplicates.

(E) Determination of alpha$_2$ platelet adrenoceptors following the method of Boon and Elliott (1981)

Anticoagulation mixture	1.0% disodium E.D.T.A 0.7% NaCl pH = 7.4
Incubation medium	0.1% (w/v) disodium E.D.T.A 150mM NaCl pH7.5

2.1 Preparation of platelet samples

40ml of venous blood was taken from an antecubital vein by clean venepuncture and with minimum stasis. The blood was immediately transferred into a plastic universal container containing the above anticoagulant mixture in a ratio of ten volumes of blood: one volume of anticoagulant.

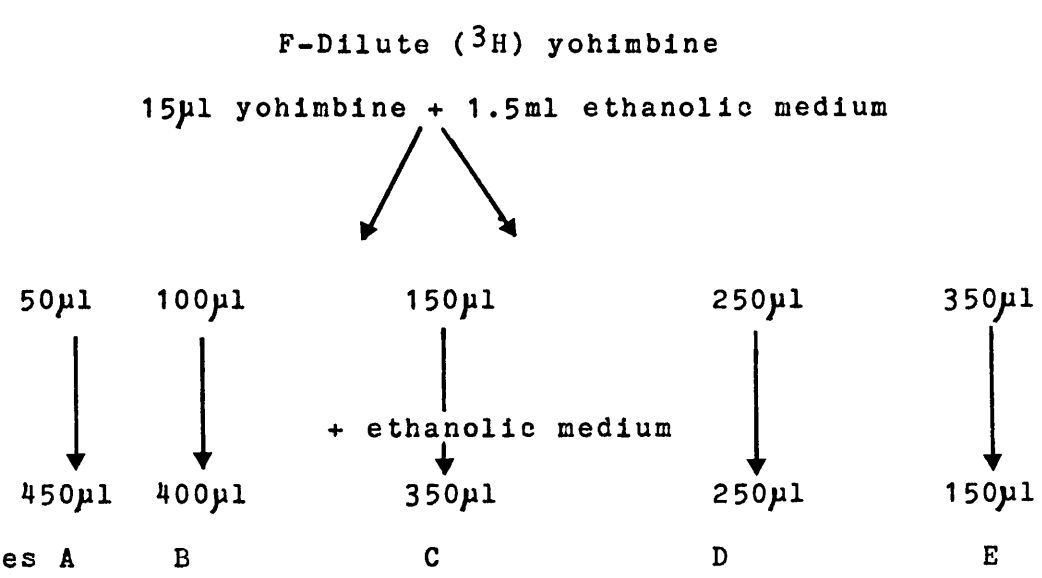
These samples were then centrifuged at 180g for 15 minutes at 20°C to obtain platelet rich plasma. The platelet rich plasma, was then decanted using plastic transfer pipettes into a second plastic universal container and further centrifuged at 1,700g for 10 minutes at 10°C. After centrifugation the plasma was discarded, and the platelets resuspended in 200µl of incubation buffer medium 0.1% (w/v disodium EDTA and 150mM NaCl pH 7.5) by use of a vortex mixer. The platelets were then diluted by further addition of incubation medium and counted in a Coulter S Plus III, adjusting the count if necessary with further additions of

incubation medium to yield platelets of approximately 10^8cells/ml.

2.2 Assay of alpha$_2$ platelet adrenoceptors with (^{3}H) yohimbine

The experimental design was as follows:-

The stock solution of (^{3}H) yohimbine was diluted in a mixture of 90% incubation medium and 10% ethanol in a ratio of 15µl(^{3}H) yohimbine plus 1.5ml of ethanolic medium. This constitutes the highest concentration in the assay (designated F). Dilutions were then made from solution F into tubes A, B, C, D, and E using the ethanolic medium as below:-



Six sets of tubes for each dilution were made at this stage (A-F).

A solution of 50µM of Phentolamine was also prepared for the use of non specific binding in the assay.

The assay tubes were then set up each containing 50µl of the diluted (^{3}H) yohimbine, six samples at each dilution (A-F). To three samples at each concentration of (^{3}H) yohimbine 50µl of incubation medium was added and to the other three samples at each concentration, 50µl of phentolamine solution was added.

Incubations were started by adding to each of these assay tubes 400µl of platelet suspensions containing approximately 10^8cells/ml. These tubes were then incubated for twenty minutes at 37°C in a shaking water bath. Incubations were terminated by centrifuging the assay tubes at 6,000g for two minutes at 20°C.

A 100µl sample of the supernatant from each assay tube was removed carefully and added to a scintillation vial containing 5ml of scintillation fluid (Insta-gel), these were then counted in a scintillation counter to estimate the free concentration of (^{3}H) yohimbine in the incubation.

Any remaining supernatant was poured off and 500µl of distilled water added to each assay tube and sonicated to resuspend the platelet pellet. 400µl of this mixture was then taken from each tube, put into a scintillation vial with 5ml of scintillation fluid (Insta-gel) and again counted. The three tubes in the assay containing (^{3}H) yohimbine and incubation medium measure total binding.

The three tubes in the assay containing (^{3}H) yohimbine and phentolamine measure the non specific binding.

Specific binding of (^{3}H) yohimbine = total counts - non specific binding counts.

Scatchard plots (Scatchard 1949). This is a manipulation of the "specific binding" data used to produce a linear equation. The relationship between bound and free radioligand is hyperbolic (assuming a single binding site)

$$B = \frac{F \times Bmax}{F + Kd}$$

B - Specific Bound radioligand

F - Free concentration radioligand

Bmax - Maximum binding capacity (number of receptors)

Kd - Dissociation equilibrium constant (receptor affinity).

Scatchard plot is

$$\frac{B}{F} = \frac{Bmax}{Kd} - \frac{B}{Kd}$$

So by plotting B/F against B, the result is a straight line with the slope = 1/Kd and the x - intercept = Bmax.

The radioligand binding was analysed by Scatchard plot analysis using linear regression with all assays having correlation coeficient greater than 0.9 (for plots of Bound/Free V.S. Bound (^{3}H) yohimbine.

Results were expressed as mean $\pm$ S.D. and affinity (Kd) and capacity (Bmax) calculated from the Scatchard plots.

(F) In Vitro platelet studies with thiol reactive compounds

2.1 D-pen

Normal citrated platelet rich plasma (PRP) suspensions were prepared as described in (B) 2.1 and platelet aggregation as in (B) 2.2.

The platelet suspensions were adjusted to $250 \times 10^9/l$ from healthy normal controls (no drugs) and divided into 1ml aliquots.

The experimental design was as follows:

(a) 1ml PRP + 5µg/ml D-pen) 10 mins
(b) 1ml PRP + 10µg/ml D-pen) incubation
(c) 1ml PRP + 20µg/ml D-pen) 37^0C
(d) 1ml PRP - no D-pen control)

These experiments were repeated at 30mins and 60mins incubation respectively.

Following the incubations of the platelet suspensions with the three drug concentrations of D-pen, they were immediately stimulated with 100µl of (a) ADP 2.5µmol/l, (b) collagen 4µg/ml, (c) adrenaline 5µmol/l and examined for aggregation for up to five minutes at 37^0C.

The capacity of each agonist to either induce or inhibit platelet aggregation was calculated from the aggregomoter tracings and recorded as a percentage.
Each of the drug concentrations were studied on platelets coming from the same donor, thus eliminating incidental and individual variations in platelet aggregability.

2.2 Sodium aurothiomalate

Experimental design was as follows:

(a) 1ml PRP + 1.5µg/ml aurothiomalate) 10mins
(b) 1ml PRP + 3µg/ml aurothiomalate) incubation
(c) 1ml PRP + 5µg/ml aurothiomalate) 37^0C
(d) 1ml PRP - no aurothiomalate control)

The above experiments were repeated at 30mins and 60mins incubations and at the above drug concentrations. The experimental design was then exactly as for D-pen as described in (F) 2.1.
Percentage aggregation to each agonist was calculated from the aggregometer tracings and recorded.

2.3 phydroxymercuriphenylsulphonate (pHMPSA) + L-cysteine

pHMPSA an irreversible non penetrating SH blocking agent and L-cysteine - a sulphydryl containing compound able to compete with the cellular sulphydryl sites for the pHMPSA on

platelets were used in this in vitro system. Working solutions of pHMPSA 10^{-4}M and L-cysteine 10^{-4}M were prepared fresh for each set of experiments.

The citrated platelet rich plasma (PRP) suspensions were prepared as described in (B) 2.1 and platelet aggregation as in (B) 2.2.
The platelet suspensions were adjusted to $250x10^{9}$/l from healthy normal controls (no drugs) and divided into four 1ml aliquots.

The experimental design was as follows:

(a) 1ml Control PRP - no additions)1 minute
(b) 1ml PRP + 10^{-4}M pHMPSA)incubation
(c) 1ml PRP + 10^{-4}M L-cysteine) 37^{0}C
(d) 1ml PRP + 10^{-4}M pHMPSA + 10^{-4}M L-cysteine)

Following the incubation at 37^{0}C of the normal platelet rich suspensions (PRP) with pHMPSA 10^{-4}M and L-cysteine 10^{-4}M either singly or in combination they were immediately stimulated with 100µl of (a) adrenaline 5µmol/l, (b) collagen 4µg/ml (c) ADP 2.5 µmol/l and examined for aggregation at 5mins, 10mins, 15mins, 20mins, 25mins, and 30mins at 37^{0}C.

The capacity of each agonist on the platelet suspensions to either induce or inhibit aggregation was calculated from the aggregometer tracings and reported as a percentage.

2.4 Dithiothreitol (DTT)

DTT a potent reducing agent was used in this in vitro system. The stock solution was kept for one week at 4^0C as a 100mM solution in isotonic saline, working solutions of 0.5mM and 1.0mM were prepared fresh in isotonic saline for each experiment.

The citrated platelet rich plasma (PRP) suspensions were prepared as described in (B) 2.1 and platelet aggregation as in (B) 2.2.

The platelet suspensions were adjusted to 250 x 10^9/l from both rheumatoid patients receiving daily doses of D-pen and normal healthy controls, then split into 3 and 2 aliquots (1ml) respectively.

The experimental design was as follows:

(a) 1ml RA - D-pen PRP - no DTT) 1 minute
(b) 1ml RA - D-pen PRP + 0.5mM DTT) incubation
(c) 1ml RA - D-pen PRP + 1.0mM DTT) 37^0C
(d) 1ml Control PRP + 0.5mM DTT)
(e) 1ml Control PRP - no DTT)

These experiments were repeated at 2mins and 5mins incubation respectively.

Following the various incubations at 37^0C the above platelet suspensions were immediately stimulated with 100µl of

adrenaline 5µmol/l and examined for aggregation for up to 5mins at 37^0C.

The capacity of 5µmol/l adrenaline on the platelet suspensions to either induce or inhibit platelet aggregation was calculated from the aggregometer tracings and reported as a percentage.

(G) Groups of patients studied

2.1 Spot studies

2.1.1 Normal controls:- n=20

These were twenty laboratory staff: ten male and ten female, mean age 31.5 years, range 18-45 years, not taking any drugs, oral contraceptives and free from intercurrent and chronic disease. All were sero-negative for classical rheumatoid factor.

2.1.2 Chronic inflammatory disease:- n=20

These were twenty patients: twelve female and eight male, mean age 41.6 years, range 18-74 years, and were sero-negative for classical rheumatoid arthritis. Their disease states included ulcerative colitis, Crohns disease and ankylosing spondylitis. Treatment included steroids, sulphasalazine or azathioprine.

2.1.3 Rheumatoid patients NSAIDs:- n=20

These were twenty patients 8 male and twelve female, mean age 45.5 years, range 22-70 years, receiving NSAIDs but no second line treatment with D-pen. All patients were sero-positive for classical rheumatoid factor.

2.1.4 Rheumatoid patients treated with D-pen:- n=20

These were fifteen females and five males, mean age 50.5 years, range 25-74 years, receiving doses of D-penicillamine 375mg-750mg/day All patients were sero-positive for classical rheumatoid factor.

2.2 Serial studies

2.2.1 Normal controls:- n=20

These were the twenty laboratory staff used in the spot studies.

2.2.2 Rheumatoid patients NSAIDs:- n=20

These were 11 females and 9 males mean age 47.3 years, range 27-72 receiving NSAIDs, (Naprosyn, Voltarol, Flurbiprofen, Ibuprofen, Sulindac and Indomethacin), no second line treatment with D-pen. All patients were sero-positive for classical rheumatoid factor.

2.2.3 Rheumatoid patients Pre-D-pen (untreated -0 months):- n=20

These were twenty patients: fourteen female and six male, mean age 47.9 years, range 28-62 years, having received no second line treatment with D-pen. All patients were sero-positive for classical rheumatoid factor.

2.2.4 Rheumatoid patients (treated with D-pen 0-18 months):- n=20

The same twenty of the above group after two and three months treatment with D-pen (final dose 375mg-750mg/day) and examined at two, four, six, eight, twelve, fourteen, sixteen and eighteen months.
Any patient receiving aspirin was excluded from the study.

(H) Measurement of disease activity (rheumatoid arthritis)

2.1 Assessment

The activity and severity of the disease was assessed by an Activity Index compiled using, morning stiffness, grip strength, articular index, onset of fatigue and ESR as described by Wallace & Ragan (1958). See Table A. This protocol was also used to identify responders and non-responders to D-pen therapy, a fall of 1 or more in the activity index being regarded as a positive response.

OVERALL R.A. ACTIVITY SCORE	INACTIVE 1	MILDLY ACTIVE 2	ACTIVE 3	VERY ACTIVE 4
DURATION OF MORNING STIFFNESS	None	Less than 30 mins	30 mins - 3 hrs	Greater than 3 hrs
ARTICULAR INDEX (RITCHIE)	None	Less than 6	6 - 20	Greater than 20
GRIP STRENGTH	Greater than 280mm.Hg.	220 - 280 mm. Hg.	150 - 220 mm.Hg.	Less than 150mm.Hg.
ONSET OF FATIGUE	None	Slight	Moderate	Pronounced
ESR	Less than 20mm/hr	20-35mm/hr	35-50mm/hr	Greater than 50mm/hr

TABLE A Assessment of Disease Activity in Rheumatoid Arthritis

These values were used as guidelines for allocating patients to an overall activity score. The assessment also included analysis of functional status (Steinbrocker) and anatomical stage (Radiological).

2.2 Grade of activity

Patients were then graded as:-

Inactive	=	1
Mildly active	=	2
Active	=	3
Very active	=	4

All controls were graded '0' as they were all sero-negative for classical rheumatoid factor.

The assessment of the activity of the disease in the patient was carried out by one Medical Officer, therefore, removing as far as possible, any discrepancies in the scoring.

(I) Induction regime of D-pen

2.1 Starting dose

Starting dose 125mg x 2:-	250mg/day
after 1 month 125mg x 3:-	375mg/day

2.2 Maintenance dose

after 2 months either remain:-	375mg/day
or 125mg x 4:-	500mg/day
occasionally higher to a maximum:-	750mg/day

(J) Statistical methods

2.1 Mean values, standard deviation, linear regression and coefficent of variation were calculated according to conventional methods (Armitage, 1977).

2.2 Statistical analysis:- Mann-Whitney-U-Test. (Mann & Whitney, 1947).

Data obtained from patients in this study were not normally distributed. Therefore, non-parametric tests (Mann-Whitney-U-Test) were used to compare results from the various patient and control groups. This method involves ranking data points from the 2 groups being compared and summing the ranks for each set. The smaller rank total is compared with values published in tables for differing sample sizes. In this thesis, the Mann-Whitney-U-Test was carried out using an Interstat programme on an Apple IIe microcomputer.

CHAPTER THREE:

RESULTS - SPOT STUDIES

3.1 Comparison of haemoglobin, mean platelet volume, total platelet count and platelet crit in (a) healthy controls, (b) RA-NSAIDs, (c) RA-D-pen treated, (d) inflammatory disease states

In a wide series of observations the platelet count in healthy individuals remains within a fairly narrow range of 150 - $400x10^9/l$ and the day-to-day individual variations are minimal. This study was designed to compare the haemoglobin and platelet parameters in a group of patients with rheumatoid arthritis with an observed thrombocytosis (range $357-740x10^9/l$) subsequently treated with D-pen and with healthy controls and other RA patients NSAIDs treated and with a group of inflammatory disease states.

Haemoglobin and platelet parameters

The methods used to derive the haemoglobin and platelet parameters are refinements of the well established Coulter principle of counting and sizing in combination with an automatic diluting and mixing device for sample processing and a single beam photometer for haemoglobinometry:- the Coulter S Plus III and data terminal. Calibration, linearity, accuracy, reproducibility and precision were maintained throughout all the experiments on the Coulter S Plus III by performing strict quality control on reagents and standards and in measuring the haematological parameters in triplicate

between one and three hours and recording the means.

The Coulter S Plus III measured the haemoglobin as cyanmethaemoglobin in gms/dl at 525 nanometers, the absorbance of which was directly proportional to the haemoglobin concentrations of the sample in the test system. This compares to the reference material for haemoglobin recommended by the International Committee for Standardisation in Haematology, (ICSH, 1978).

Platelet volume analysis used in this study was also performed on the Coulter model S Plus III, an instrument which separates platelets by differential gating measured by electrical impedance and uses the log normal principle to derive the platelet parameters. For total platelet count, mean platelet volume and platelet crit the Coulter S Plus III produced logarithmic transformation which generated data that fitted the Gaussian distribution.

The haemoglobin values for the various patient groups and controls are shown in Fig.3.1.a Two hundred and forty Coulter counter measurements were made from the four groups studied and in all three disease states the mean haemoglobin concentration was significantly lower than the control group ($P < 0.001$).

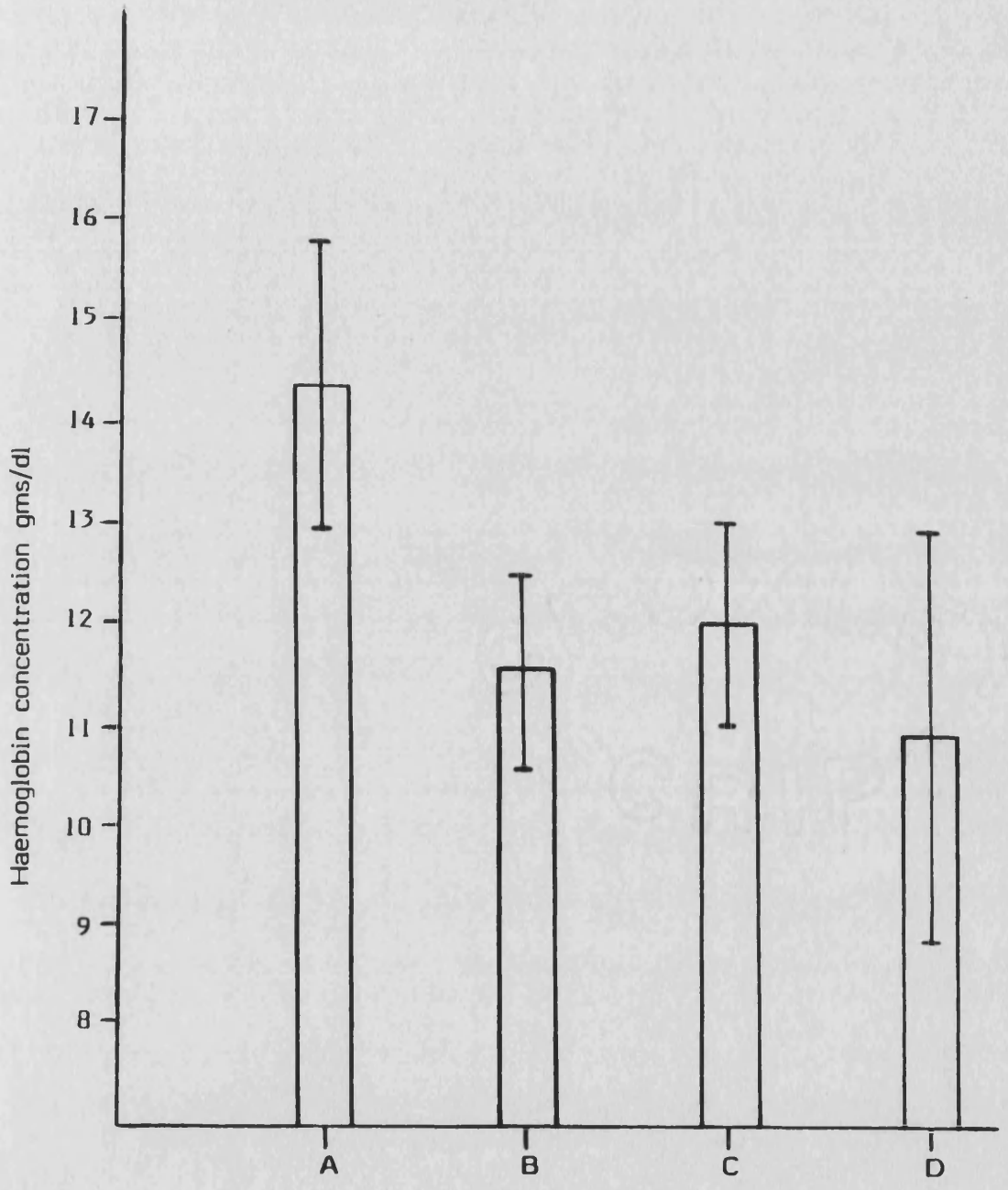


Fig. 3.1.a Haemoglobin levels gms/dl in (A) Normal Controls, (B) R.A. NSAIDs treated, (C) R.A. D-pen treated, (D) Chronic Inflammatory diseases. The results are expressed as mean $\pm$ S.D. (n = 20). Statistical analysis shows:- a highly significant difference between A vs B, C & D ($P < 0.001$).

The results in Fig.3.1.b and Table 1 show the mean level of platelet counts within the four groups investigated. Compared to the normal control mean $288\pm54x10^9/l$ both rheumatoid groups showed a significant elevated mean platelet count. $500\pm88x10^9/l$ and $461\pm142x10^9/l$ respectively ($P < 0.001$, $P < 0.001$). There was no significant difference between normal controls and the platelet counts in the inflammatory disease group.

Mean platelet volume analysis

Mean platelet volumes measured in femtolitres (fl) were also studied and these results are illustrated in Fig 3.1.c and Table 1. The mean platelet volume in the RA groups, RA-NSAIDs 7.5 ± 0.7 fl; RA-D-pen 7.8 ± 0.9 fl were significantly lower than the MPV of the control group 9.3 ± 0.9 fl, or the inflammatory disease group, 9.3 ± 1.2 fl ($P < 0.001$), RA-NSAIDS and ($P < 0.001$) RA-D-pen. However, there was a considerable overlap between the various groups. Histograms producing platelet volume distribution were plotted using an X-Y recorder (results not shown) from data generated from the Coulter S Plus III. Variables of platelet volume analysis were detected by measuring their relative surface area in the histograms, then calculating the percentage of microthrombocytes and megathrombocytes present. Microthrombocytes were defined as particles with a volume between 2-5fl and megathrombocytes as particles with a volume greater than 13fl.

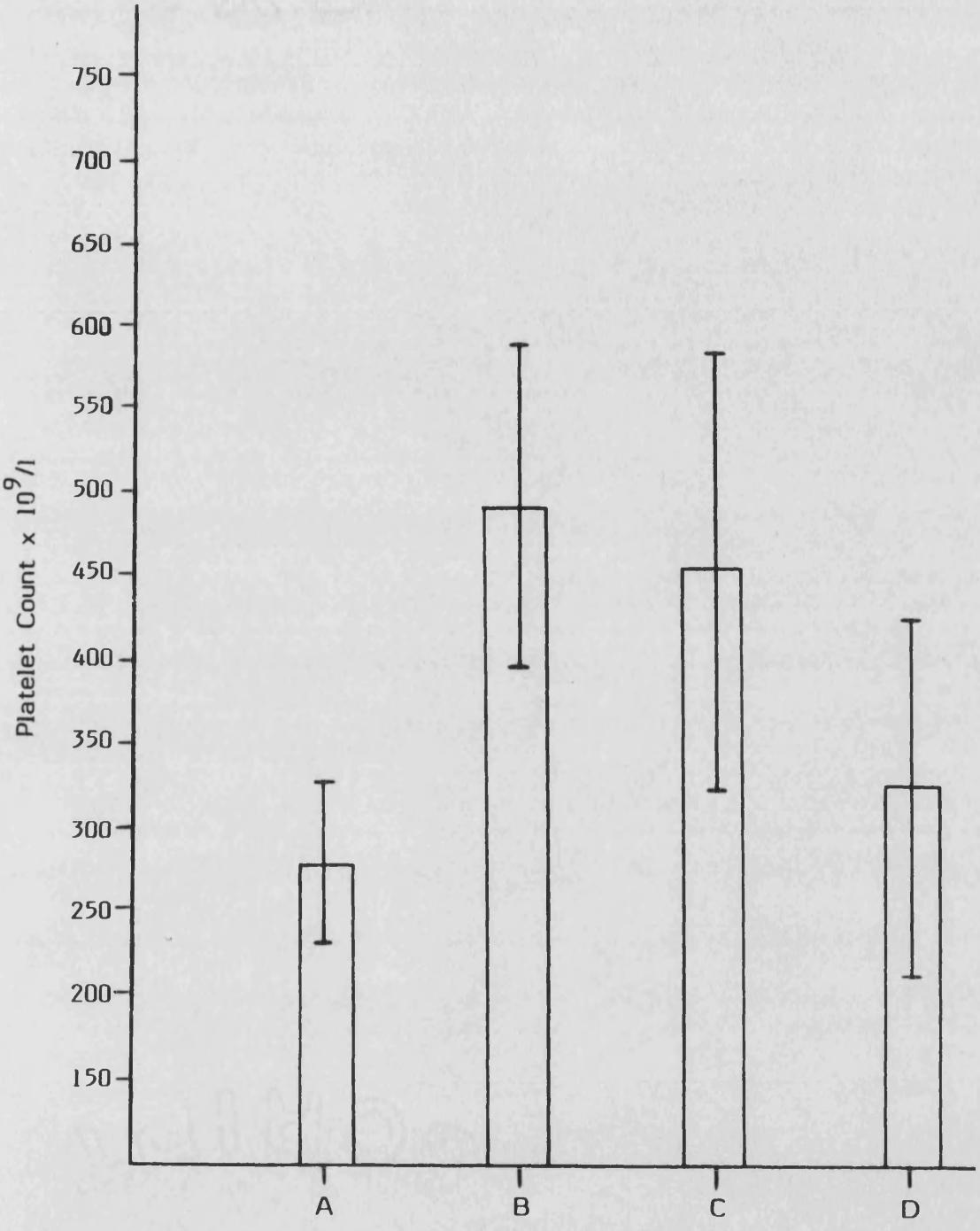


Fig. 3.1.b Platelet counts x 10^9/l in (A) Normal Controls, (B) R.A. NSAIDs treated, (C) R.A. D-pen treated, (D) Chronic inflammatory diseases. The results are expressed as mean ± S.D. (n = 20). Statistical analysis shows:- a highly significant difference between A vs B ($P < 0.001$) and A vs C ($P < 0.001$).

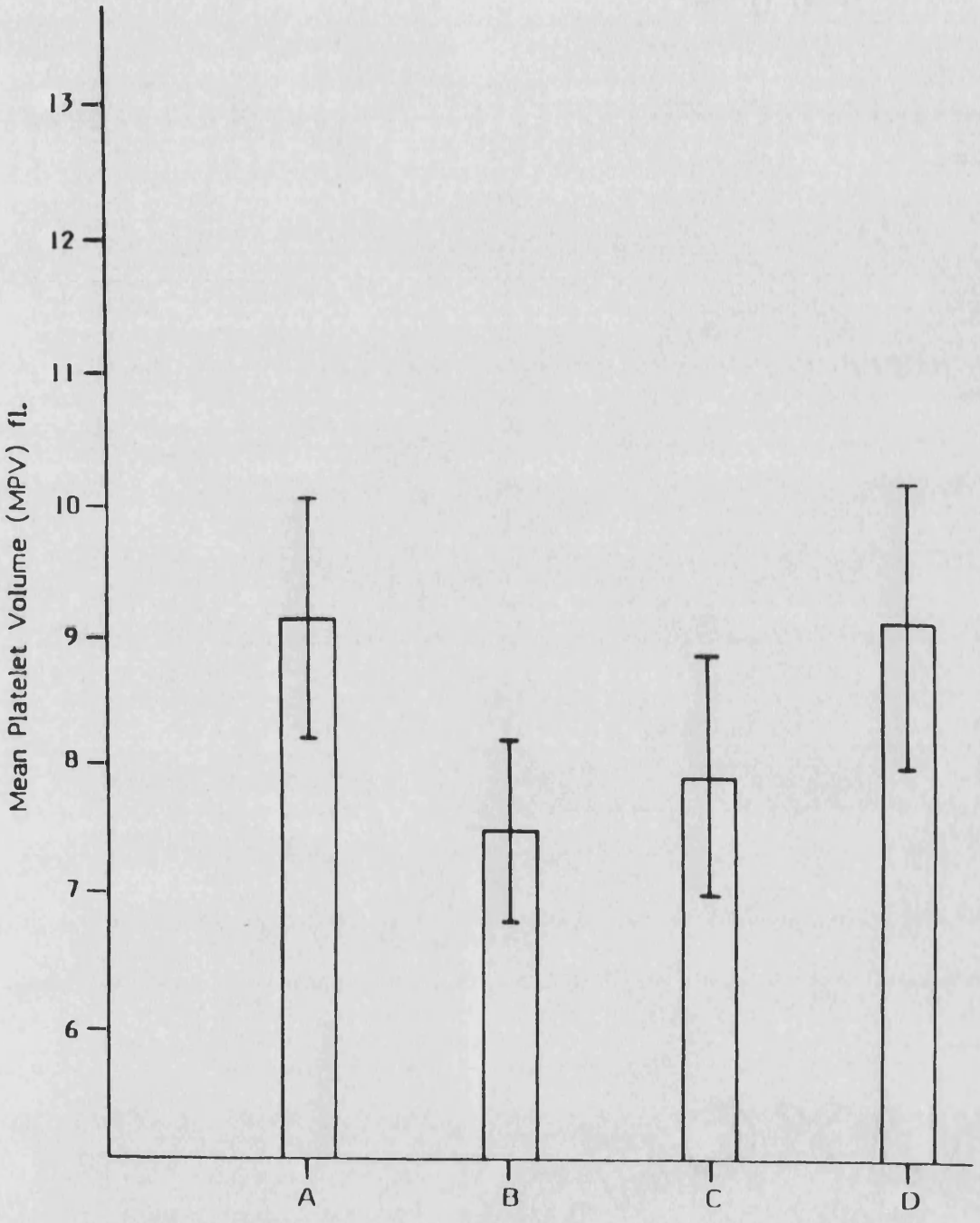


Fig 3.1.c Mean platelet volume (MPV) fl. in (A) Normal Controls, (B) R.A. - NSAIDs treated, (C) R.A. D-pen treated, (D) Chronic inflammatory diseases. The results are expressed as mean ± S.D. (n = 20). Statistical analysis shows:- a highly significant difference between A vs B ($P < 0.001$) and A vs C ($P < 0.001$).

PATIENT GROUPS	NORMAL CONTROLS	RA-NSAIDs	RA-D-PEN	INFLAMMATORY DISEASES
Plt Count x 10^9/l	288 ± 53	500 ± 88*	461 ± 142**	338 ± 123
MPV(fl)	9.3 ± 0.9	7.5 ± 0.7*	7.8 ± 0.9**	9.0 ± 1.2
PDW	16.4 ± 0.7	16.2 ± 0.5	16.1 ± 0.4	16.2 ± 0.6
Plt Size 2-5fl%	22.5 ± 3.7	31.4 ± 4.4*	32.1 ± 4.2**	21.2 ± 3.9
Plt Size	8.8 ± 1.8	4.2 ± 2.1*	4.5 ± 2.0**	6.9 ± 2.6

TABLE I Variables of platelet volume analysis in normal subjects and different patient groups. The results are expressed as mean ± S.D. (n=20). Statistical analysis shows:- a highly significant difference between Normal vs RA-NSAIDs* ($P < 0.001$) and Normal vs RA-D-pen** ($P < 0.001$).

Table 1 shows the results of determining the percentage of microthrombocytes 2 - 5fl and megathrombocytes 13 - 20fl from the platelet volume distribution histograms. Compared with normal healthy subjects, the RA group with thrombocytosis had a substantially increased number of small platelets, mean $32.1 \pm 4.2\%$ compared to normal controls $22.5 \pm 3.7\%$ ($P < 0.001$) and fewer larger platelets, mean $4.5 \pm 2\%$ compared with controls $8.8 \pm 1.8\%$, these results show that the percentage of megathrombocytes in rheumatoid patients was significantly lower than in normal subjects. (Table 1) ($P < 0.001$).

Platelet crit

As shown in Fig.3.1.d the third platelet parameter investigated in this study was the platelet crit %. Any expansion of the platelet count and the mean platelet volume is an expression of the platelet crit. The platelet count is the prime determinant in the platelet crit and an abnormality in the MPV produces only comparatively minor differences. Platelet crit was calculated from the following formula:-

$$\text{Platelet crit } \% = \frac{\text{Plt count} \times \text{MPV}}{10{,}000} \%$$

In the four groups of patients studied, the rheumatoid groups displaying thrombocytosis had significantly higher values of

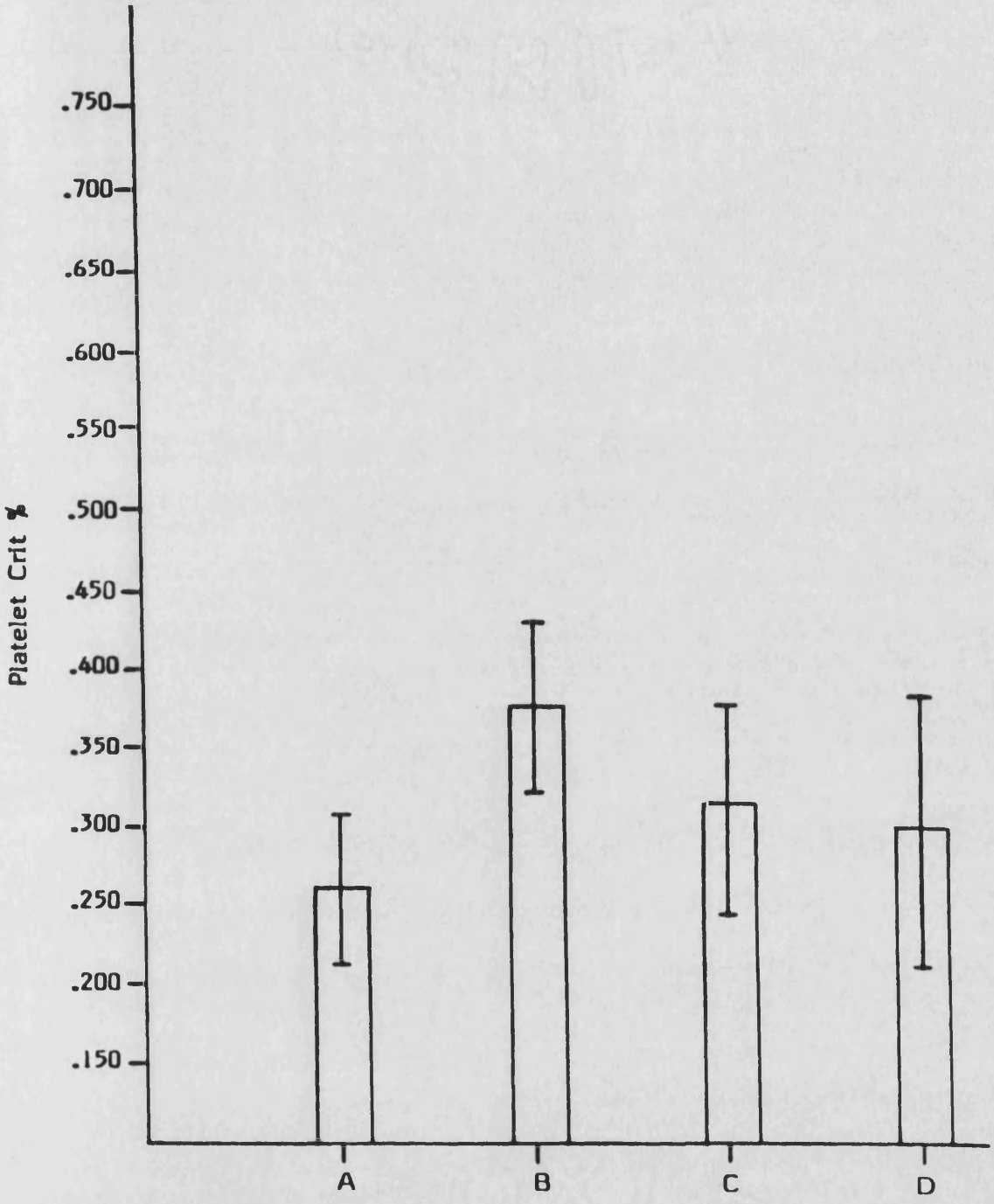


Fig. 3.1.d Platelets Crit (%) in (A) Normal Controls, (B) R.A. NSAIDs treated, (C) R.A. D-pen treated, (D) Chronic Inflammatory diseases. The results are expressed as mean ± S.D. (n = 20). Statistical analysis shows:- a highly significant difference between A vs B ($p < 0.001$) and A vs C ($P < 0.001$).

platelet crit, 0.378±0.060% NSAIDs, and 0.338±0.084% RA-D-pen compared to the normal controls 0.268±0.055% ($P < 0.001$). The inflammatory disease group did not differ significantly from the control group, mean 0.300±0.065%. There were no differences between groups of patients in terms of age or sex, and these factors did not influence the platelet parameters measured.

3.2 Platelet aggregation with ADP, adrenaline and collagen

Platelets are known to aggregate under a variety of conditions and in the presence of a number of different reagents. For conventional aggregation ADP, adrenaline and collagen were extensively used. For the purpose of comparison, the changes in light transmission during the first five minutes of aggregation was used for maximum aggregation results with the three platelet agonists. This protocol gave more consistent results, as the first two minutes could vary considerably between individual samples and with the particular agonists used.

Quantitation of Aggregation results

Changes in %Transmission (T) of various PRPs during aggregation were recorded with the aggregation controls adjusted to give 100% T with water in the cuvette and zero T

with no input into the chart recorder.

The recorded T values were then treated in the following manner. The apparatus was adjusted so that PRP and PPP read 0% and 100% T respectively. To facilitate comparison with this approach the recorded T values were converted to T^1 values by the following equation:-

$$T^1\% = \frac{T(\%) - T(\%)\ (PRP)}{T(\%)(PPP) - T(\%)(PRP)} \times 100$$

T(%) (PRP) is the T% value of the PRP before the addition of the agonist. The rate of aggregation was then recorded as the maximal increase in T(%) over the five minute time interval.

Typical representative traces are shown in Fig.3.2.a.

3.2.1 Illustrations of actual traces of normal platelet aggregation to agonists ADP, adrenaline and collagen

Primary aggregation or the first wave of aggregation refers to the direct aggregation of platelets by adenosine diphosphate (ADP) and other aggregating agents. Primary aggregation is accompanied by shape changes and is a reversible process, this is normally followed by a secondary

or second wave of aggregation. Secondary aggregation is irreversible and is accompanied by the extrusion of the content of the granules found in the platelet cytoplasm. For the purpose of comparison the changes in transmittance of the platelet suspension with various concentrations of the agonists were measured for up to 5 mins for maximum aggregation and the percentage aggregation calculated from the results of the tracings.

Aggregation by ADP

Increasing concentrations of ADP were added to aliquots of the same platelet rich plasma (0.01 µmol/l - 4.0 µmol/l ADP) and Fig.3.2.a records a typical family of traces. It will be seen that aggregation begins at once and with increasing concentrations the initial slope increases indicating more rapid aggregation. The initial slope of these curves which are almost linear are proportional to the log of the dose of ADP added. With concentrations of ADP between 0.2 µmol/l and 0.5 µmol/l a primary wave was observed followed by disaggregation, however, concentrations of ADP above 1.0µmol/l produced the secondary wave of aggregation which blended with the primary wave forming a single broad wave of aggregation. This at 2.0 µmol/l concentration represented approximately 50% platelet aggregation. When ADP concentrations were increased to 4.0 µmol/l maximum aggregation was achieved.

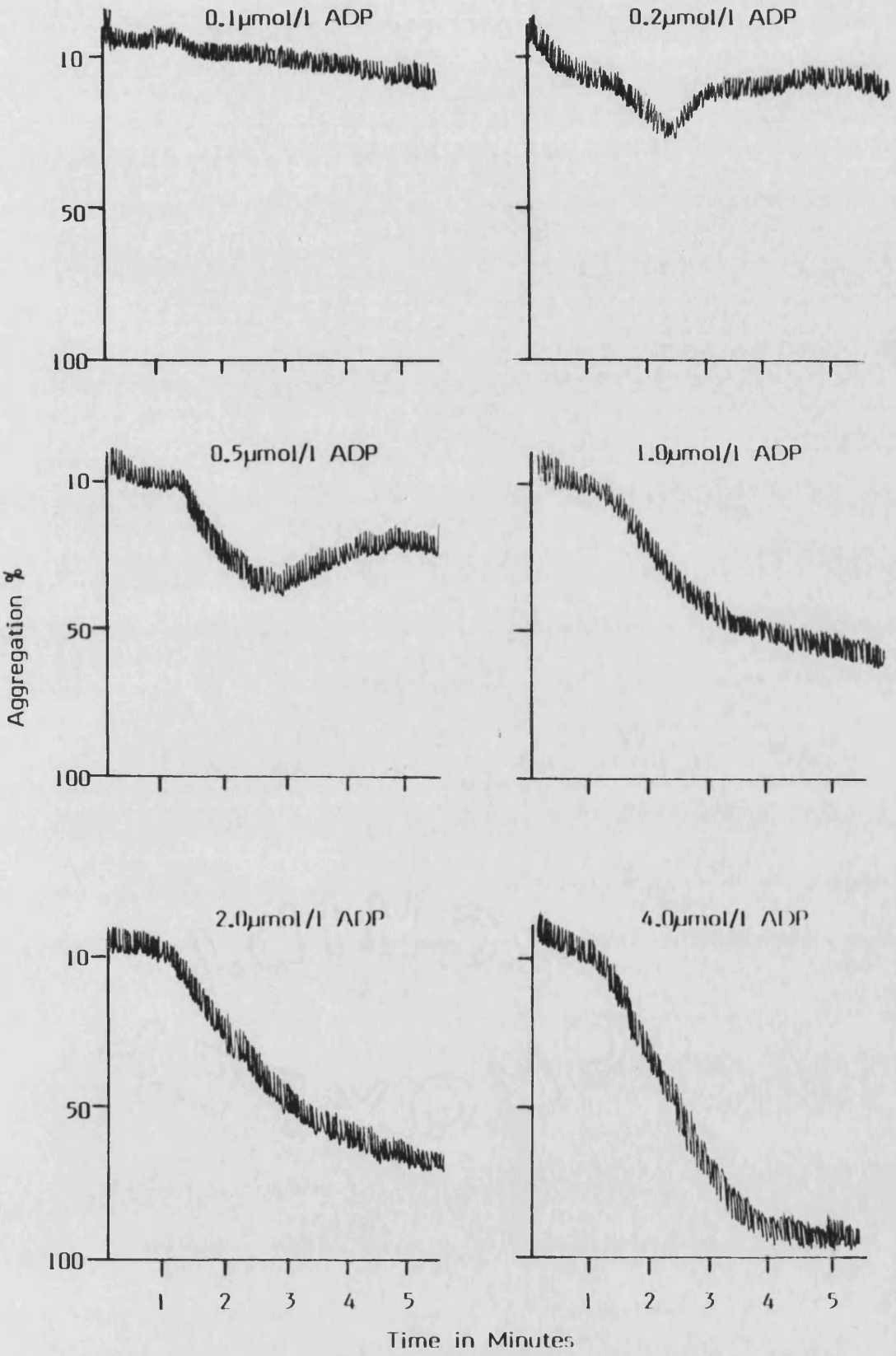


Fig. 3.2.a Aggregometer tracings of ADP induced aggregation by increasing concentrations of agonist.

Aggregation by adrenaline

Inspection of the traces in Fig.3.2.b produced by adding increasing concentrations of adrenaline (0.1 µmol/l -8.0 µmol/l) to aliquots of the same platelet rich plasma shows that aggregation began immediately and that the initial slope depends on the concentration of the adrenaline added, as with ADP, the slope was proportional to the log of the dose.
With smaller doses 0.1 µmol/l - 1.0 µmol/l aggregation ceased after about 70 seconds, a time that was constant over this range, but some disaggregation occurred.
Following this plateau there was a delay of several minutes which appeared to be dose dependent. Aggregation increased, became rapid, and was always complete and irreversible.

Thus adrenaline concentrations <0.4 µmol/l induced only a weak reversible aggregation response. However, with concentrations ranging between 1.0 µmol/l and 8.0 µmol/l the aggregatory response was monophasic and irreversible reaching approximately 50% aggregation between 2.0 µmol/l and 4.0 µmol/l with maximum aggregation at a concentration of adrenaline 8.0 µmol/l (see Fig 3.2.b).

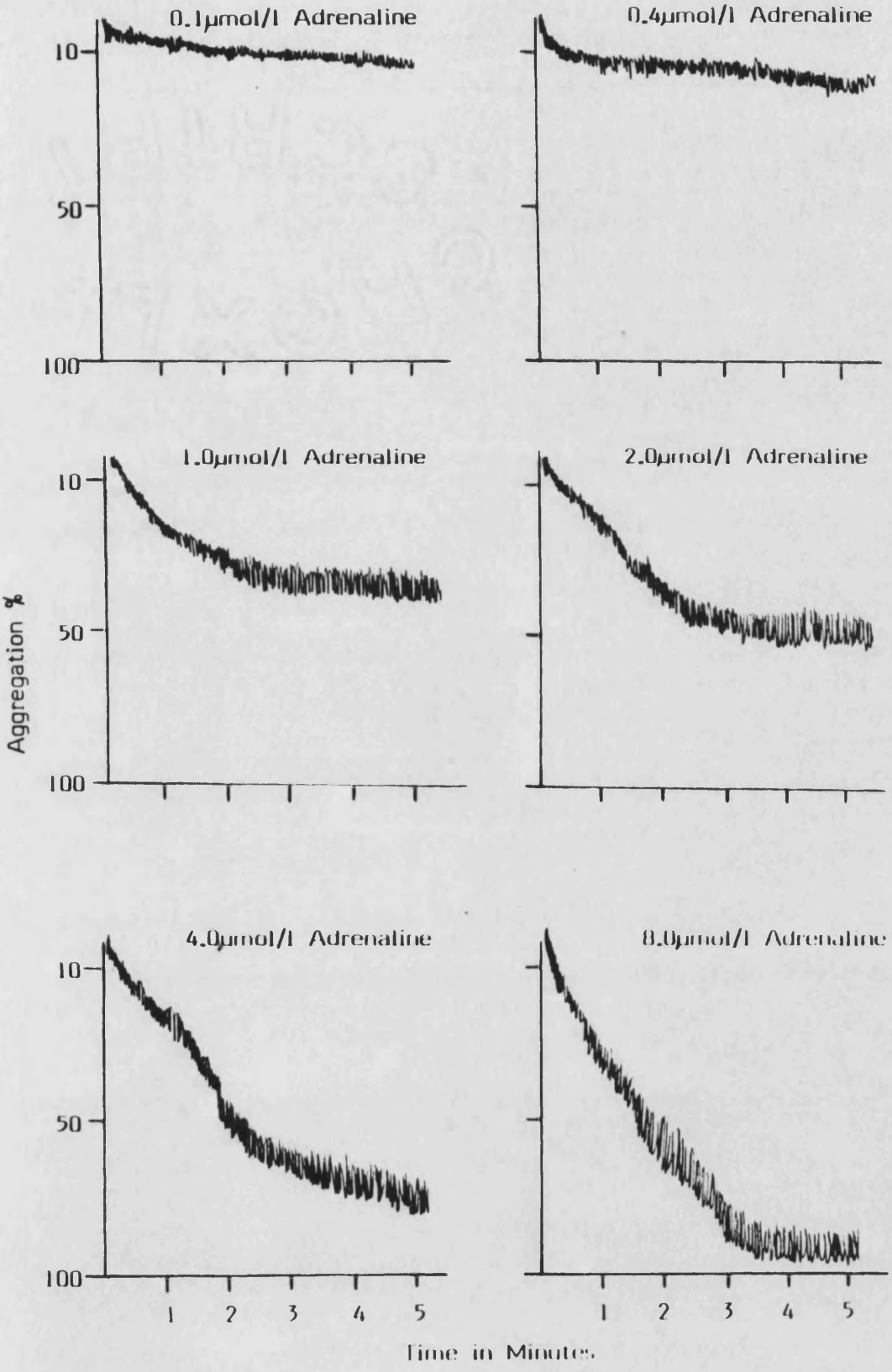


Fig. 3.2.b. Aggregometer tracings of Adrenaline induced aggregation by increasing concentrations of agonist.

Aggregation by collagen

Collagen induces only a secondary wave of aggregation which corresponds to the release reaction of the platelets.

A typical collagen curve was characterised by a lag phase before aggregation occurred. Typical aggregation traces are shown in Fig.3.2.c. At concentrations of collagen between 0.2 -0.4 µg/ml no aggregation was observed. However, between 0.8 µg/ml and 1.6 µg/ml concentration there was a delay period of 1 - 2 minutes (the lag phase) followed by aggregation to approximately 50%. As the concentration of collagen was increased between 4.0 - 8.0 µg/ml aggregation correspondingly increased to a maximum level. The range of concentrations used in this study were later utilised to determine the ED_{50} for each of the agonists studied in the various patient groups.

3.3 Representative sigmoid dose response curves and calculation of ED_{50} results, showing aggregation responses to increasing concentrations of ADP, adrenaline and collagen within the various patient groups

In order to establish the optimum dose of agonist needed for platelet aggregation in the various disease states dose response curves were produced for each individual within that group and plotted versus increasing concentrations of the agonist. By examining the curves and plotting concentration

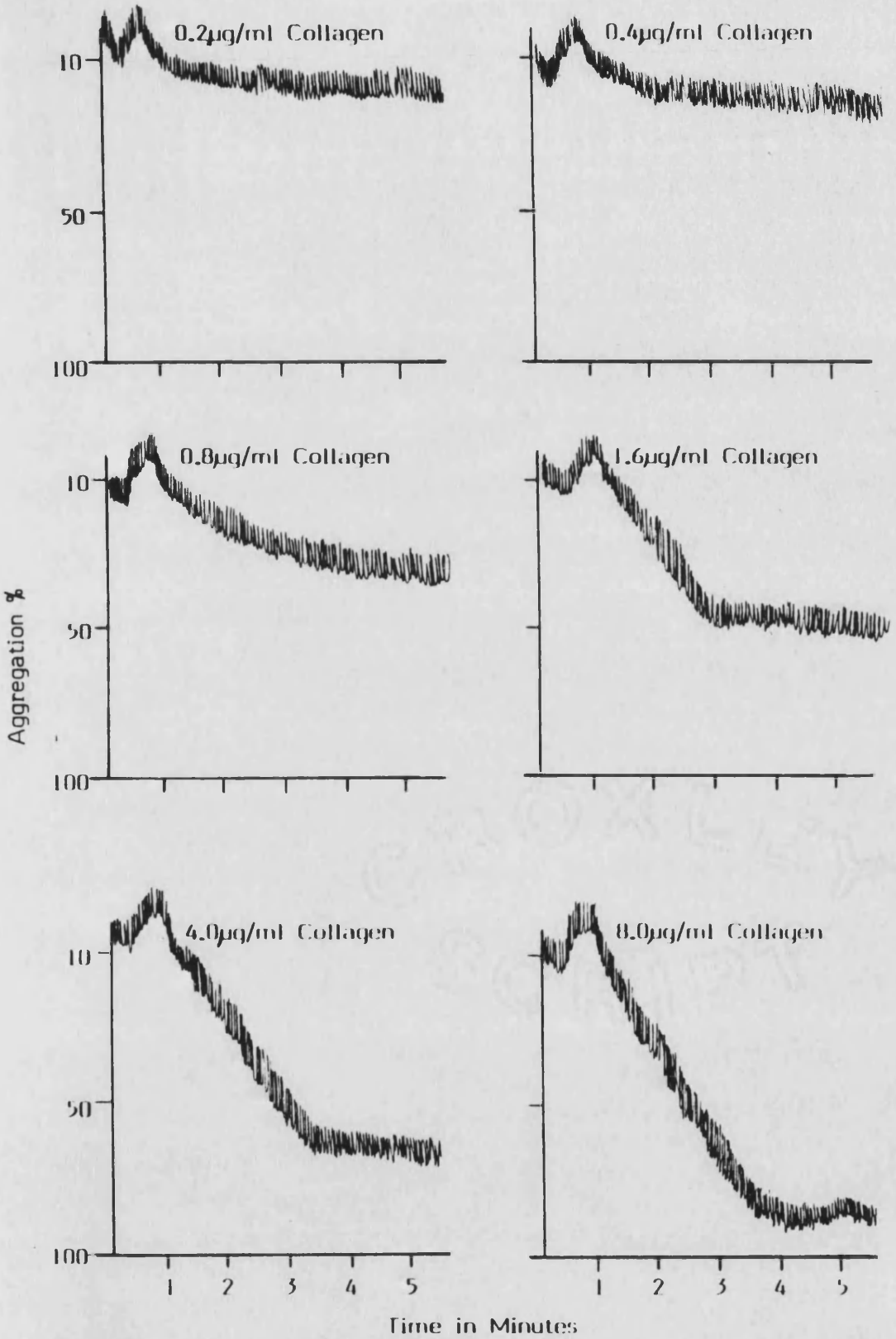


Fig. 3.2.c Aggregometer tracings of Collagen induced aggregation by increasing concentrations of agonist.

versus aggregation, ED_{50} values for each agonist could be produced thus allowing standardisation within the experiments. Following this procedure these conditions would then allow the test system to detect any possible increases or decreases of sensitivity to platelet aggregation with ADP, adrenaline, and collagen within control and patient groups studied.

ADP

Increasing concentrations of ADP (0.1 μmol - 4.0 μmol/l) were added to aliquots of the various platelet rich plasmas and Fig.3.3.a illustrates typical sigmoid representative curves for (a) healthy normal controls, (b) RA-NSAIDs (c) RA-D-pen treated and (d) chronic inflammatory diseases. Platelets from both the rheumatoid groups showed increased sensitivity (RA-NSAIDs > RA-D-pen treated) to low concentrations of ADP. When the ED_{50}'s were calculated and plotted as shown in Fig.3.3.b there were significant differences in ADP concentrations required for 50% aggregation in controls versus RA-NSAIDs ($P < 0.005$), RA-NSAIDs versus RA-D-pen ($P < 0.05$), RA-NSAIDs versus inflammatory disease states ($P < 0.01$). The ranges of ADP ED_{50} concentrations in all groups overlapped considerably, normal controls 0.3 μmol - 1.5 μmol/l, RA-NSAIDs 0.15 μmol - 1.0 μmol/l, RA-D-pen 0.15 μmol - 1.5 μmol/l, and inflammatory group 0.3 μmol - 1.5 μmol/l. There was no correlation between platelet sensitivity to ADP and age or sex within the patient groups.

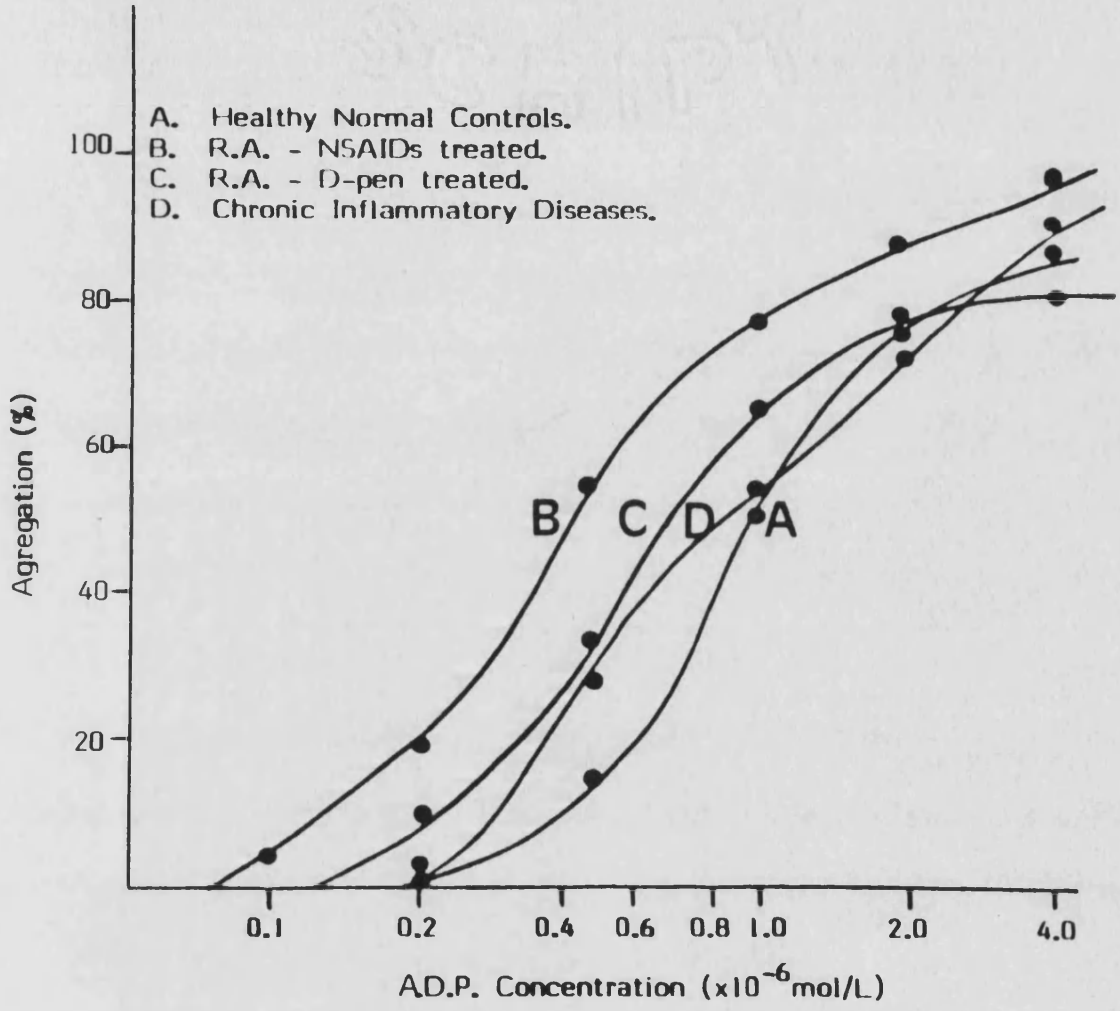


Fig.3.3.a Representative curves showing aggregation responses to increasing concentrations of A.D.P.

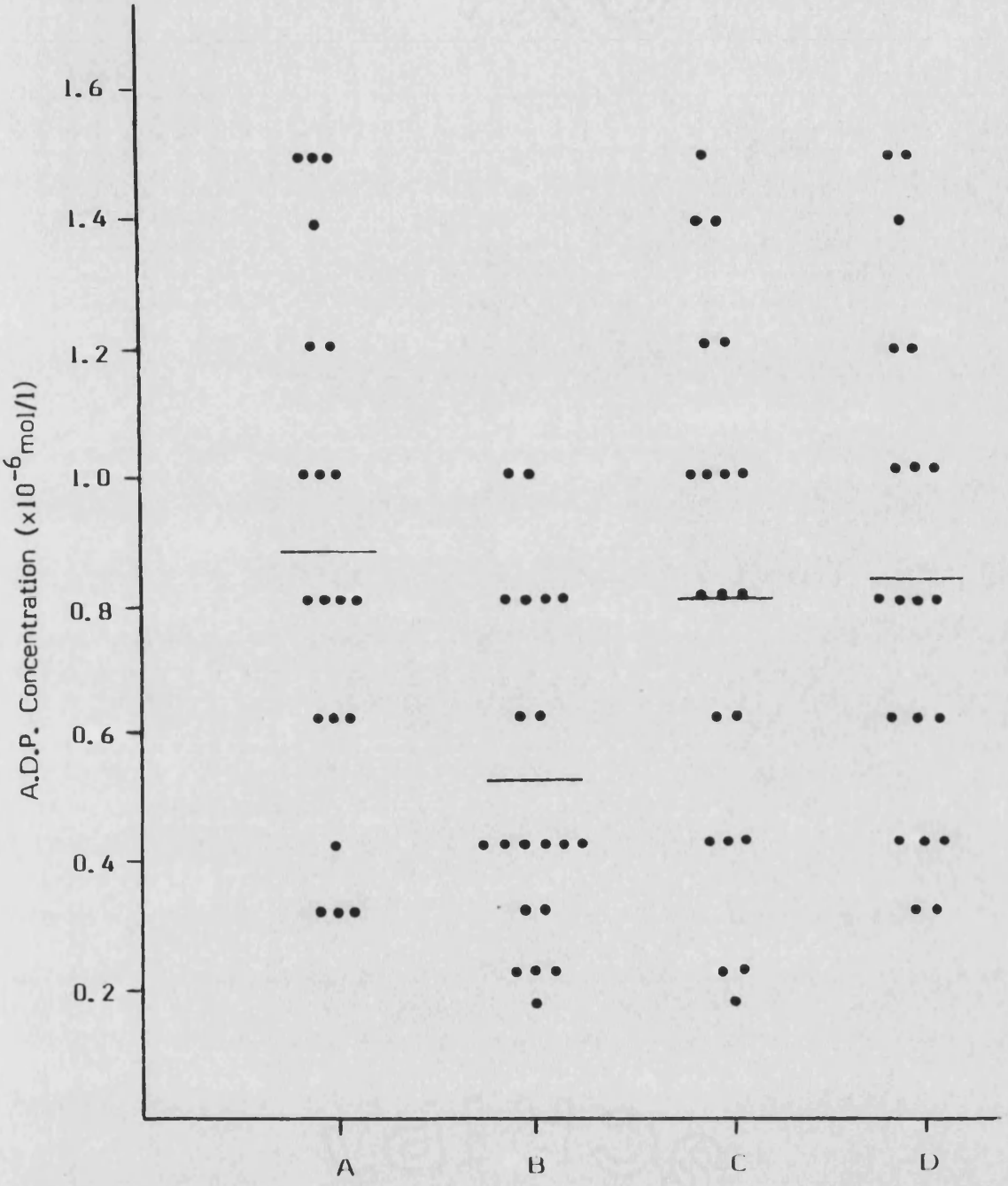


Fig 3.3.b A.D.P. Concentrations inducing 50% aggregation (ED_{50}) in (A) Normal Controls, (B) R.A. - NSAIDs treated. (C) R.A. D-pen treated, (D) Chronic Inflammatory disease. Bars indicate means (n = 20). Statistical analysis shows:- a significant difference between A vs B ($P < 0.005$); B vs C ($P < 0.05$) and B vs D ($P < 0.01$).

Adrenaline

Increasing concentrations of adrenaline (0.1 μmol - 100 μmol/l) were added to the patient group platelets and these results are shown in Fig.3.3.c. The dose response curve indicates that there was a dramatic decrease in adrenaline stimulated responses with rheumatoid platelets treated with D-pen. Even with concentrations of 100 μmol/l adrenaline, the dose response curve was shifted far to the right, with a total loss of sensitivity to adrenaline. When compared with the normal group, RA-NSAIDs and inflammatory disease groups showed a slight increase in sensitivity to adrenaline.

The ranges of adrenaline ED_{50} concentration overlapped in three of the groups i.e. normal controls 0.2 μmol - 5.0 μmol/l, RA-NSAIDs 0.1 μmol - 4.0 μmol/l, and inflammatory disease group 0.1 μmol - 5.0 μmol but there was no overlap in the RA-D-pen treated group.

Two patients only showed some aggregation at 10 μmol/l but the remaining eighteen patients failed to aggregate, even at a concentration of 100 μmol/l adrenaline. This was obviously highly significant ($P < 0.001$). There was no correlation between sensitivity to adrenaline and age or sex in the patient groups.

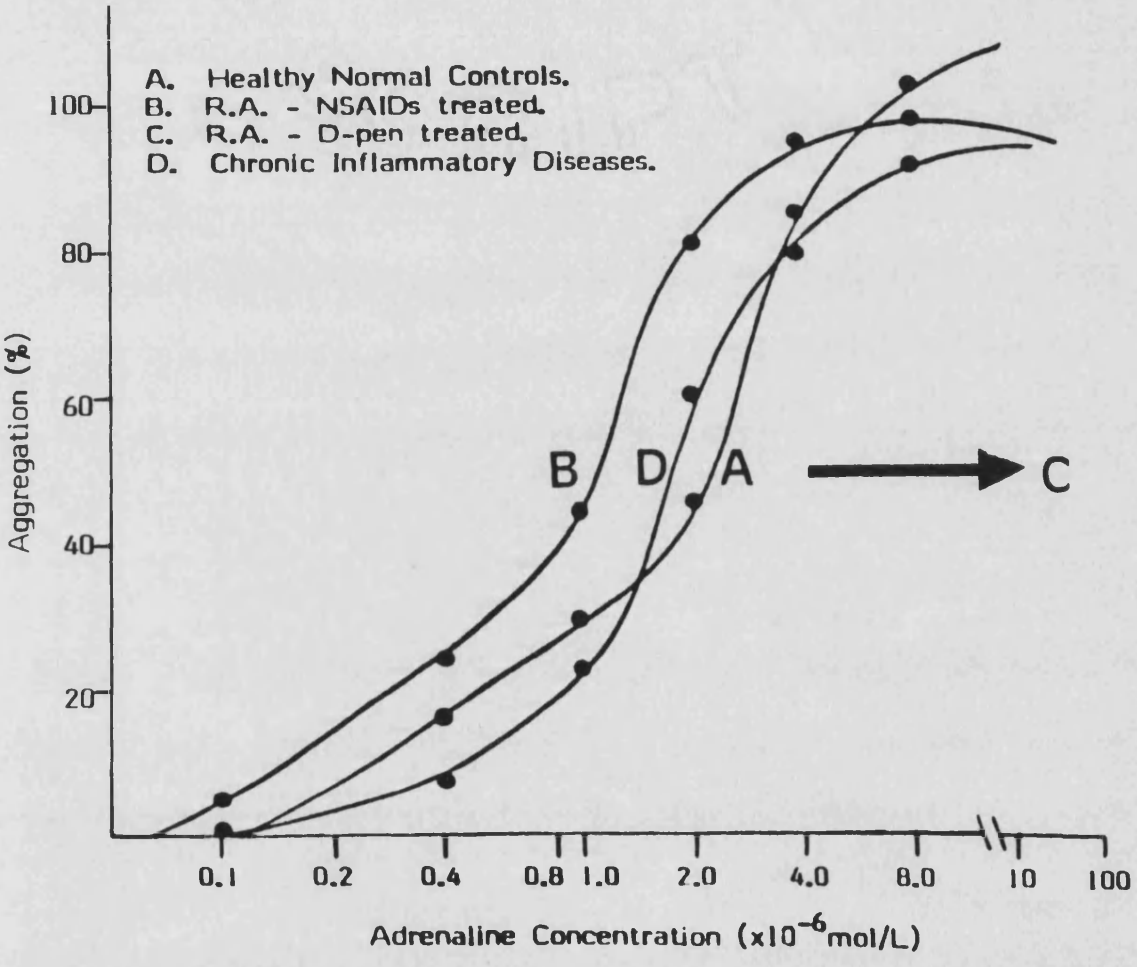


Fig.3.3.c Representative curves showing aggregation responses to increasing concentrations of adrenaline.

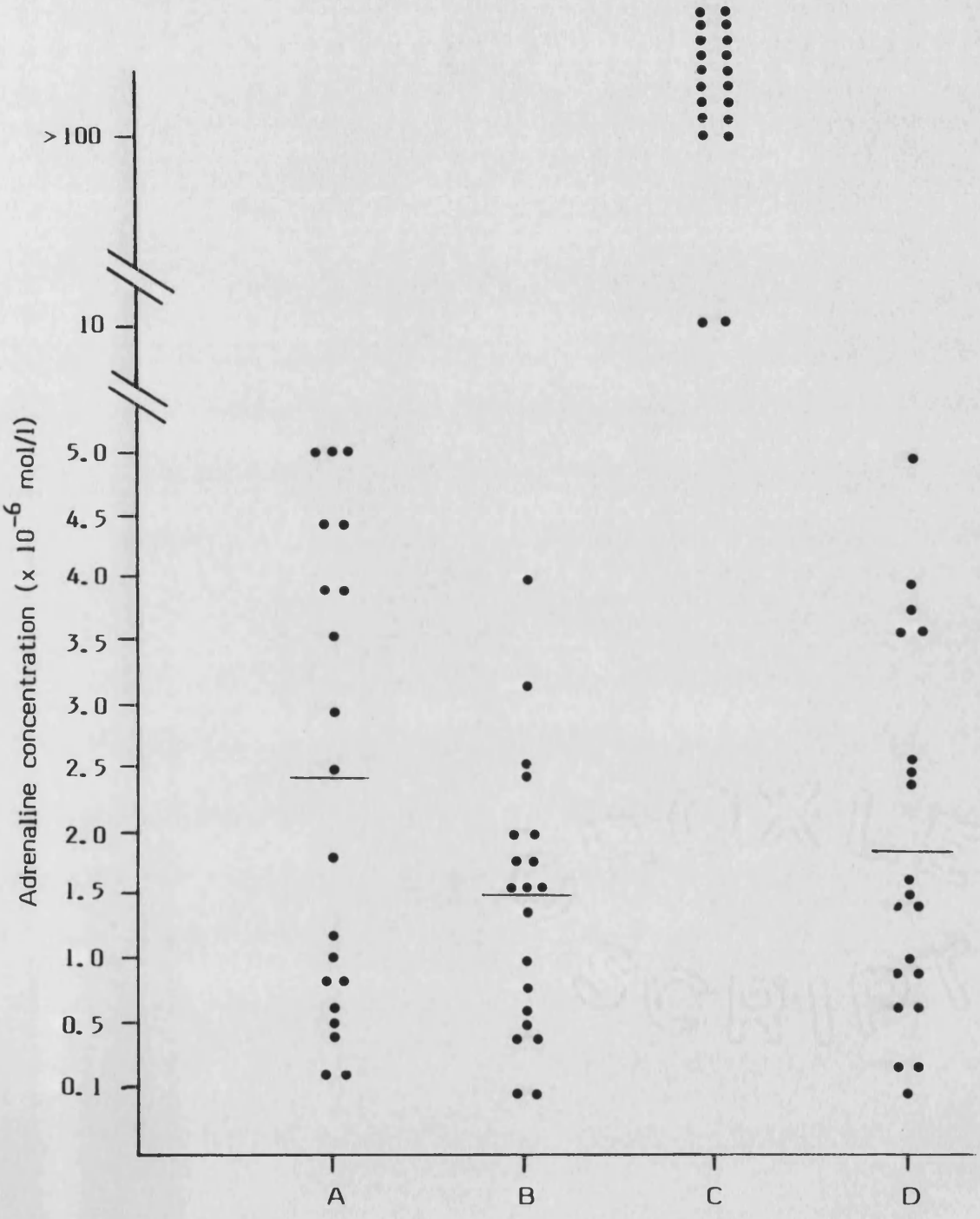


Fig. 3.3.d Adrenaline concentration inducing 50% aggregation (ED_{50}) in (A) Normal Controls, (B) R.A. - NSAIDs treated, (C) R.A. D-pen treated, (D) Chronic inflammatory disease. Bars indicate means (n = 20). Statistical analysis shows:- a highly significant difference between C vs A, B & D ($P < 0.001$).

Collagen

As illustrated in Fig.3.3.e increasing concentrations of collagen (0.2 µg - 8.0 µg/ml) were added to aliquots of platelet rich plasma from the various patient groups and dose responses calculated for collagen gave sigmoid dose response curves.

Platelets from both the rheumatoid groups showed increased sensitivity to low concentrations of collagen (< 1.5 µg/ml). The results of the ED_{50} plots for collagen shown in Fig.3.3.f indicate a significant decrease in the amount of collagen required for 50% aggregation with RA-NSAIDs compared with any group. (Controls versus RA-NSAIDs ($P < 0.001$), RA-NSAIDs versus RA-D-pen ($P < 0.001$) and RA-NSAIDs versus inflammatory diseases ($P < 0.001$).

There was slight overlapping of the ranges of collagen ED_{50} values in all groups. Results for controls (2.0 - 5.0 µg/ml), RA-NSAIDs (0.5 - 1.5 µg/ml), RA-D-pen (1.0 - 3.0 µg/ml) and inflammatory diseases (1.5 - 4.0 µg/ml). Again there was no correlation between age or sex in the patient groups and the sensitivity of aggregation with collagen.

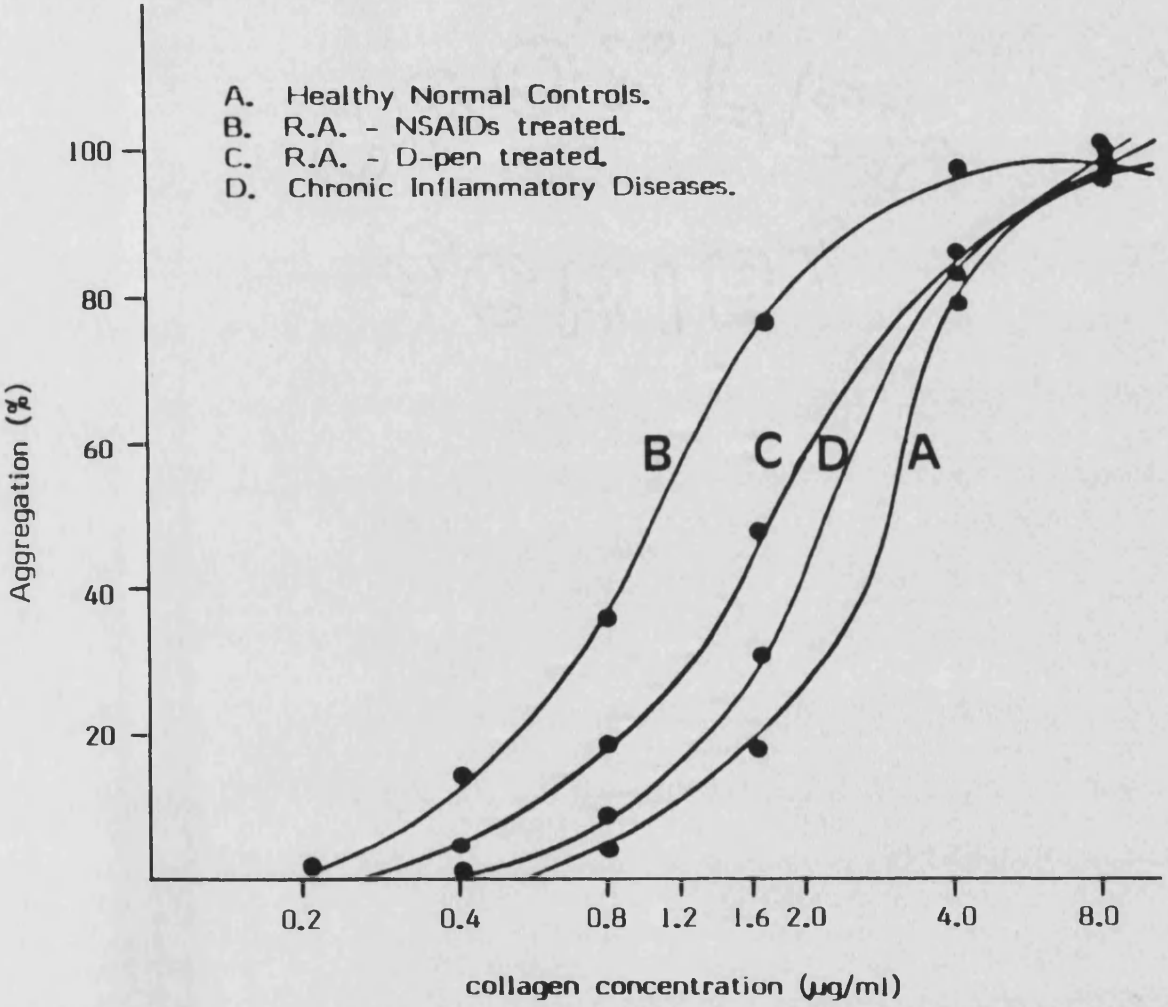


Fig. 3.3.e Representative curves showing aggregation responses to increasing concentrations of collagen.

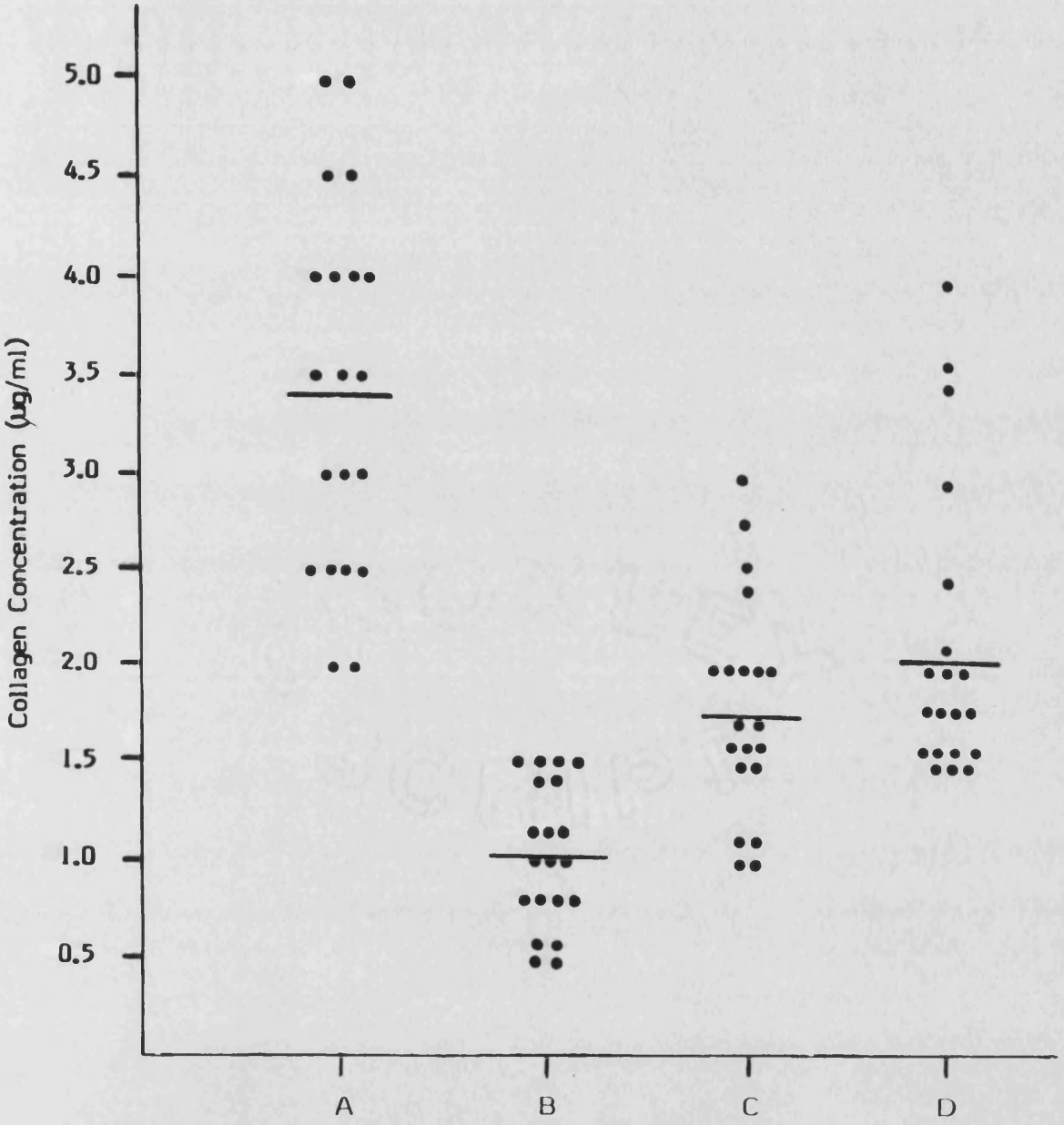


Fig. 3.3.f Collagen Concentrations inducing 50% aggregation (ED_{50}) in (A) Normal Controls, (B) R.A. - NSAIDs treated, (C) R.A. D-pen treated, (D) Chronic Inflammatory disease. Bars indicate means (n = 20). Statistical analysis shows:- a highly significant difference between A vs B ($P < 0.001$); B vs C ($P < 0.001$) and B vs D ($P < 0.001$).

CHAPTER FOUR:

RESULTS - SERIAL STUDIES

4.1 Serial Studies

Twenty patients, fourteen female and six male, mean age 47.9 years, range 28-62 years were included in the study and age-matched to a control group of healthy volunteers. The twenty patients all had classical rheumatoid arthritis and showed evidence of moderate to severe disease activity as defined by the assessment of duration of involvement, presence of extra-articular manifestations, morning stiffness, grip strength and joint score. Prior to entry, patients had been taking a variety of non-steroidal anti-inflammatory drugs (NSAIDs) but none had previously received specific anti rheumatoid drug therapy (i.e. D-pen or gold). During the initial month all patients were treated on daily doses of 250 mg followed at month 2 by 375 mg/daily and finally with a range of (250-750 mg/day) depending on clinical assessment over an 18 month period. The patients were followed at 2 monthly intervals for clinical assessment and examination of laboratory parameters.

4.1.1 Effects of D-pen treatment on platelet parameters in rheumatoid patients

Serial studies showing the effects of D-pen on platelet parameters, mean platelet volume, total platelet count and platelet crit are illustrated in Fig.4.1.a. No significant difference was noted between male and female or age of the

patients in the rheumatoid group in all of the parameters studied. On examination of the individual platelet results the mean platelet volume (MPV) at 0 months was 7.5 fl. Following six months treatment with D-pen a significant increase was noted in the MPV, mean 8.5 fl ($P < 0.001$). The increase in platelet volume was maintained at this level for the full 18 month period, mean 8.6fl. As shown in Fig.4.1.a the total platelet count had a mean value of 593 x 10^9/l in the rheumatoid group at month 0, compared with normals 288 x 10^9/l ($P < 0.001$). This level of thrombocytosis had decreased significantly to a mean of 402 x 10^9/l at month 8 of D-pen treatment ($P < 0.001$) and had decreased slightly further at month 18 to a mean of 384 x 10^9/l. The control group remained within normal platelet limits during the whole series.

Similarly the platelet crit at month 0 (0.425%) had decreased at 6 months to a mean of 0.338%, compared to a normal value of 0.268%. This decrease in platelet crit % was maintained over 18 months ($P < 0.001$). There appeared to be very minimal variation in the control group for all three parameters examined over the 18 month period.

Disease activity.

Analysis was carried out in small sub-groups of patients with differing levels of disease activity. These group sizes were small so that statistical analysis shows trends rather than significant differences. These comments are also applicable to analysis of differences between RA-responders and the small group of RA-non-responders.

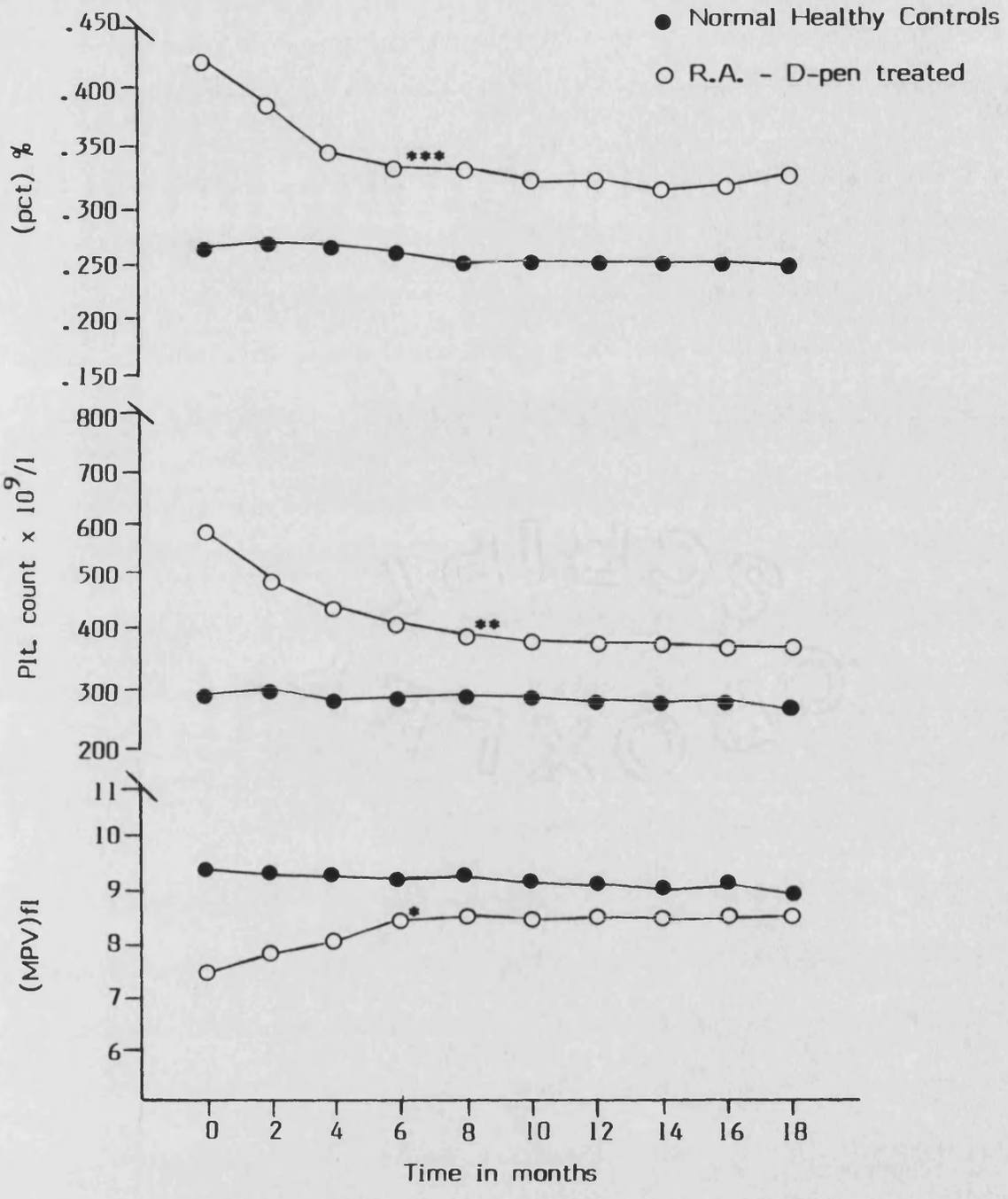


Fig.4.1.a Serial studies showing the effects on Platelet Parameters:- Mean platelet volume (MPV), Platelet count and Platelet crit % (Pct), in ● Normal Healthy Controls, ○ R.A. D-pen treated (0 - 18 months). The results are expressed as means ± S.D. (n=20). The S.D. bars are omitted for clarity. Statistical analysis shows:- a highly significant difference between Month 0 vs Month 6 R.A. D-pen* ($P < 0.001$); Month 0 vs Month 8 R.A. D-pen** ($P < 0.001$) and Month 0 vs Month 6 R.A. D-pen*** ($P < 0.001$).

4.1.2 Effects of D-pen treatment on platelets from rheumatoid patients at various stages of disease activity

The results presented in Table 2.a show that there was no significant association of platelet parameters with disease activity prior to D-pen treatment. However, post D-pen treatment all groups show changes in platelet parameters irrespective of initial score of disease activity. These results are shown in Table 2.b. All the platelet parameters differ significantly in the rheumatoid groups compared with controls, irrespective of disease activity scores.
For example, MPV values pretreatment disease activity - (2) MPV 7.82 ± 0.32 fl versus MPV post treatment 8.64 ± 0.21 fl ($P < 0.001$), similarly platelet count pre - treatment $586 \pm 59 \times 10^9$ /l versus platelet count post - treatment $385 \pm 52 \times 10^9$/l ($P < 0.001$), and thirdly platelet crit pre treatment 0.421 ± 0.018% versus post treatment 0.357 ± 0.052% ($P < 0.001$).

4.1.3 Effects of time course on D-pen dosage, ESR/hour and disease activity in rheumatoid patients

Serial changes in disease activity, mean daily dose of D-pen and ESR/hour are shown in Fig.4.2.a These were examined at two monthly intervals and up to a period of eighteen months duration in the rheumatoid group. A favourable clinical

DISEASE ACTIVITY R.A. SCORE	MEAN PLATELET VOLUME	PLATELET COUNT $x\ 10^9/l$	PLT CRIT % (Pct)
1 (n=0)	-	-	-
2 (n=5)	7.82 ± 0.32	586 ± 59	.421 ± 0.018
3 (n=4)	7.55 ± 0.31	567 ± 51	.423 ± 0.046
4 (n=11)	7.39 ± 0.70	605 ± 95	.427 ± 0.054
Control (n=20)	9.20 ± 0.87	288 ± 53	.268 ± 0.055

TABLE 2.a Pre D-Penicillamine Treatment

DISEASE ACTIVITY R.A. SCORE Pre D-pen	MEAN PLATELET VOLUME (MPV)/fl	PLATELET COUNT $x\ 10^9/l$	PLT CRIT % (Pct)
1 (n=0)	-	-	-
2 (n=5)	8.64 ± 0.21*	385 ± 52*	.357 ± 0.052*
3 (n=4)	8.40 ± 0.35*	380 ± 75*	.328 ± 0.052*
4 (n=5)	8.48 ± 0.38*	391 ± 100*	.350 ± 0.057*
Control (n=20)	9.20 ± 0.87	288 ± 53	.268 ± 0.055

TABLE 2.b Post D-Penicillamine Treatment (18 months)

Mean values of Platelet Parameters:- (MPV) Platelet Count and Pct % in R.A. patients Pre and Post D-pen treatment at different levels of disease activity, compared to controls. The results are expressed as means ± S.D. (n=20). Statistical analysis shows:- a highly significant difference between Pre vs Post D-pen treatment* ($P < 0.001$).

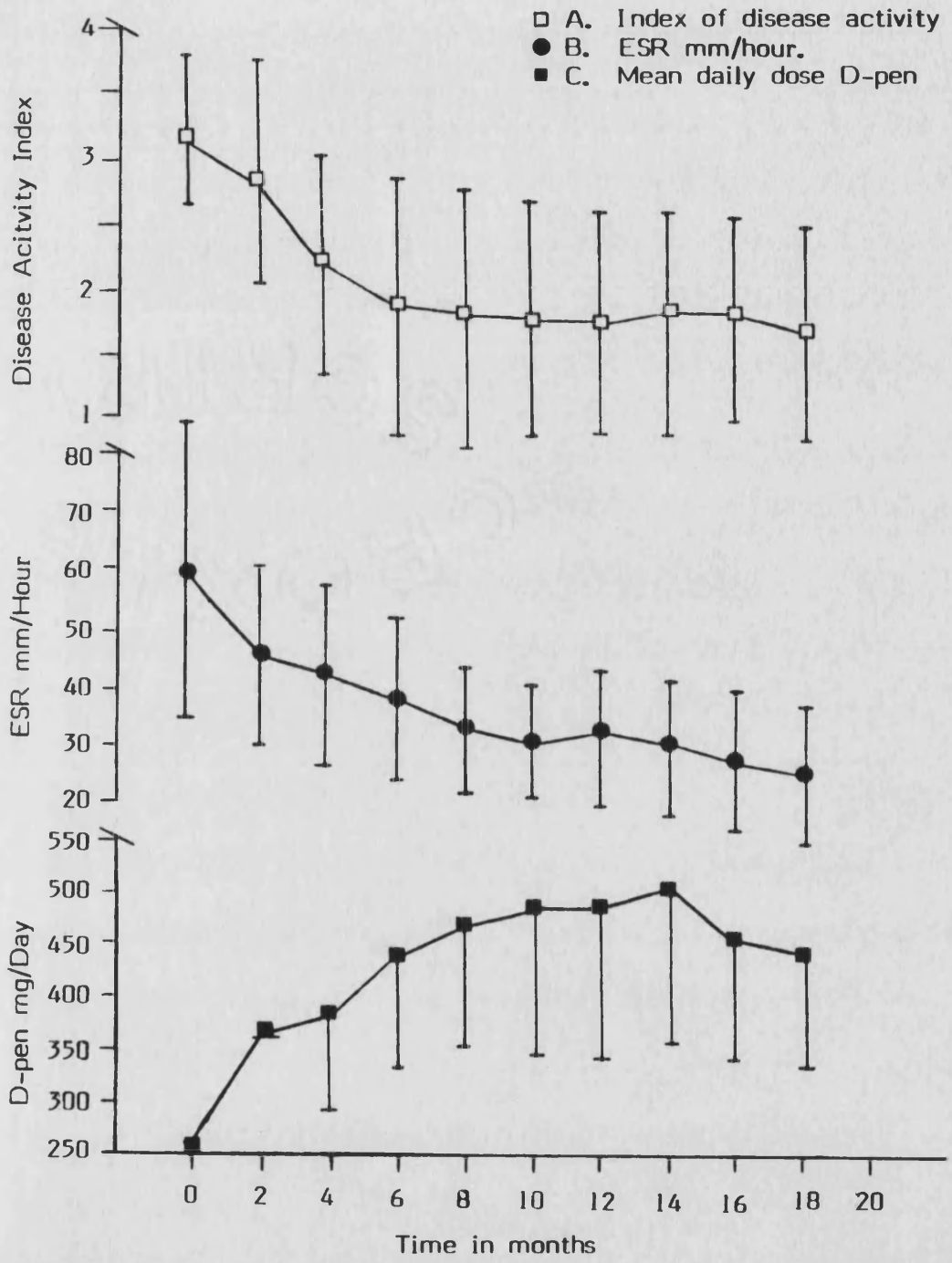


Fig.4.2.a Serial studies showing effect of time(0 - 18 months) in R.A. patients on (A) Index of disease activity, (B) Erythrocyte sedimentation rate (ESR mm/hour), (C) Mean daily dose of D-pen (mg/day). The results are expressed as means $\pm$ S.D. (n=20).

response to D-pen was observed with an index mean of 3.4 pre drug decreasing to 2.0 at six months. Although generally patients were given D-pen in increasing doses up to 750mg/day, several patients had their doses reduced because of various side effects including three cases of nausea and vomiting, four with loss of taste and one a skin rash in which the drug was stopped for a fortnight. However, restarting D-pen on a lower dose in this patient caused no further complications or recurrences. The clinical response (disease activity) was reflected by rapid improvement in the clinical measurements described in section 2.2, which reached statistical significance by 6 months compared to month 0 ($P < 0.001$). The mean level of clinical response maintained this index over the following twelve months.

The results expressed in Fig.4.2.a for the erythrocyte sedimentation rate (ESR) also showed a gradual and significant decrease from month 0 - month 18. The mean ESR pretreatment 58±25mm/hour fell by month 18 to 25±12mm/hour. This decrease was first noted after two - three months of D-pen treatment and correlated well with clinical index of disease activity.Mean daily doses of D-pen (250 - 750 mg) were either increased by 125mg/daily or correspondingly reduced by 125mg/daily depending on clinical assessment at each clinic visit and the corresponding laboratory results. These are illustrated in Fig.4.2.a Initially all rheumatoid patients commenced on levels of 250mg/daily at month 0 and

375mg/daily at month 2. There was an increase in D-pen dosage with a peak at month 14, mean 482mg/daily this very slowly decreased to month 18 with a mean daily dose of 443mg. As shown by the SD bars the standard deviations were very wide and there was marked variation between each individual studied. On clinical examination at 18 months the twenty patients in the series studied were assessed for their response to an active and fairly aggressive regime of D-pen therapy.

4.1.4 Effects of D-pen treatment on ESR/hour and platelet parameters in rheumatoid patients, showing good or poor clinical responses

After eighteen months of continual D-pen therapy clinical appraisal of the patients studied showed that out of twenty patients sixteen could be classified into responders and four into non responders. On examination of all data collected from patients and shown in Table 3 the mean daily dose of D-pen in the responder group was 406±97mg compared to the non responder group value of 593±120mg. ($P < 0.05$).
The erythrocyte sedimentation rate in the responder group had decreased with D-pen treatment to a mean of 21±8mm/hour, but remained high in the non responder group 42±7mm/hour. This compared to a pre-D-pen treatment mean at month 0 58±25mm/hour. Closer observations of platelet data derived from the responder group showed that platelet counts had decreased to values nearer those in the control group.

	RESPONDERS (n=16)	NON-RESPONDERS (n=4)
Mean Daily Dose of D-pen mg/day	406 ± 97*	593 ± 120
ESR mm/hour	21 ± 8	42 ± 7
Platelet Count $\times 10^9$/l	363 ± 73**	482 ± 29
MPV fl	8.62 ± 0.25***	8.02 ± 0.10
Platelet Crit Pct %	.331 ± 0.047****	.412 ± 0.012

Table 3 The effects of D-pen treatment in Rheumatoid Patients on E.S.R. and platelet parameters showing good or poor clinical responders. The results are expressed as mean ± S.D. Statistical analysis shows:- a significant difference between Resp vs Non-Resp* ($P < 0.05$); Resp vs Non-Resp** ($P < 0.01$); Resp vs Non-resp*** ($P < 0.01$) and Resp vs Non-Resp**** ($P < 0.01$).

Results at 18 months for the responder group were $363 \pm 73 \times 10^9/l$, compared to mean pre drug levels of $586 \pm 68 \times 10^9/l$ and non responder group $482 \pm 29 \times 10^9/l$. The change in the responder group was significantly greater than in the non responder group ($P < 0.01$).

As illustrated in Table 3 the MPV values of the responder group mean had significantly increased to 8.62 ± 0.25 fl after D-pen treatment, compared to month 0 pre-D-pen treatment MPV 7.58 ± 0.44 fl ($P < 0.001$). However, the non responder MPV remained lower at 8.02 ± 0.10 fl nearer the pre treatment value, and again this level was significantly lower than the responder group ($P < 0.01$). Similarly there was a statistical significance between platelet crit % values. Evaluation of results indicated responders to have a mean Pct of 0.331% compared to non responders 0.412% ($P < 0.01$). The platelet crit had returned after D-pen treatment much nearer the control level mean 0.268%.

4.2 Plasma Beta-thromboglobulin (B-TG) levels

Until recently relatively few methods have been available for evaluating platelet function in vivo. However the isolation and characterisation of purified platelet proteins have been followed by development of a specific and sensitive radioimmunoassay of Beta thromboglobulin (B-TG) which may be used for monitoring the platelet release reaction in vivo.

4.2.1 Calibration curve for total plasma B-TG assay

The calibration curve for total plasma B-TG is illustrated in Fig.4.3.a.It was determined by plotting the curve of ^{125}I counts for the five known concentrations of standards against the B-TG concentration. The plasma levels of experimental B-TG were estimated by reading the average of duplicate ^{125}I counts and reading off their B-TG concentrations from the calibration curve (expressed as ng/ml B-TG). A performance check at this stage was (a) to determine the time required to accumulate 10,000 counts in the 10 ng/ml standard tubes, this should not be greater than 4 minutes and

(b)

$$\text{Ratio} = \frac{\text{Mean counts in tubes 1 \& 2 (10 ng/ml)}}{\text{Mean counts in tubes 7 \& 8 (100 ng/ml)}} = >1.7$$

This ratio should be greater than 1.7 and the background counts in the assay were subtracted in calculating this ratio. The above criteria were achieved in all experiments.

4.2.2 Total plasma Beta-thromboglobulin levels in normal controls, compared with pre D-pen treated (NSAIDs) and D-pen treated rheumatoid groups

The mean plasma concentration of B-TG for controls, pre D-pen (NSAIDs) and D-pen treated are shown in Table 4.

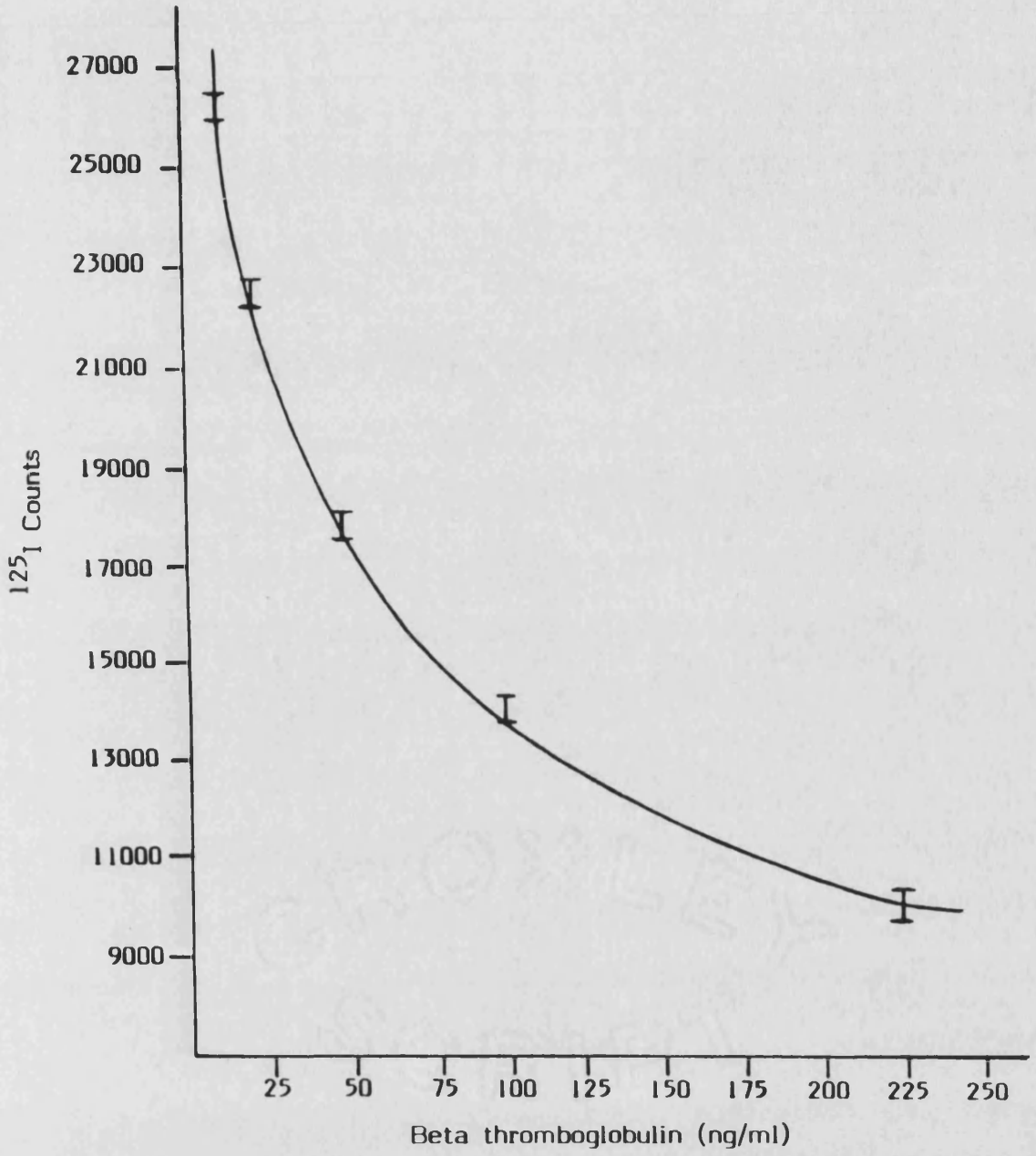


Fig.4.3.a The calibration curve for total Beta thromboglobulin assay. The results are expressed as the means of the duplicate counts.

GROUPS	BETA-THROMBOGLOBULIN LEVELS (ng/ml)	
	MEAN ± S.D.	RANGE
NORMAL CONTROLS (n=20)	32 ± 12	10 - 51
R.A. Patients Pre D-pen treatment 0 weeks (n = 20)	62 ± 30*	28 - 128
R.A. Patients D-pen treated 4 weeks (n = 11)	50 ± 17	24 - 82
R.A. patients D-pen treated 8 weeks (n = 9)	44 ± 16	30 - 78
Combined R.A. - D-pen treated 4 + 8 weeks (n = 20)	47 ± 16**	24 - 82

TABLE 4 Beta-thromboglobulin levels of normal controls, R.A. patients Pre D-pen treatment Week 0, R.A. patients D-pen treatment Week 4, R.A. D-pen treatment Week 8 and combined data of Weeks 4+8 D-pen treated. The results are expressed as mean ± S.D.
Statistical analysis shows:- a significant difference between Control vs R.A. Pre D-pen* ($P < 0.001$) and R.A. Pre D-pen vs Post D-pen** ($P < 0.05$).

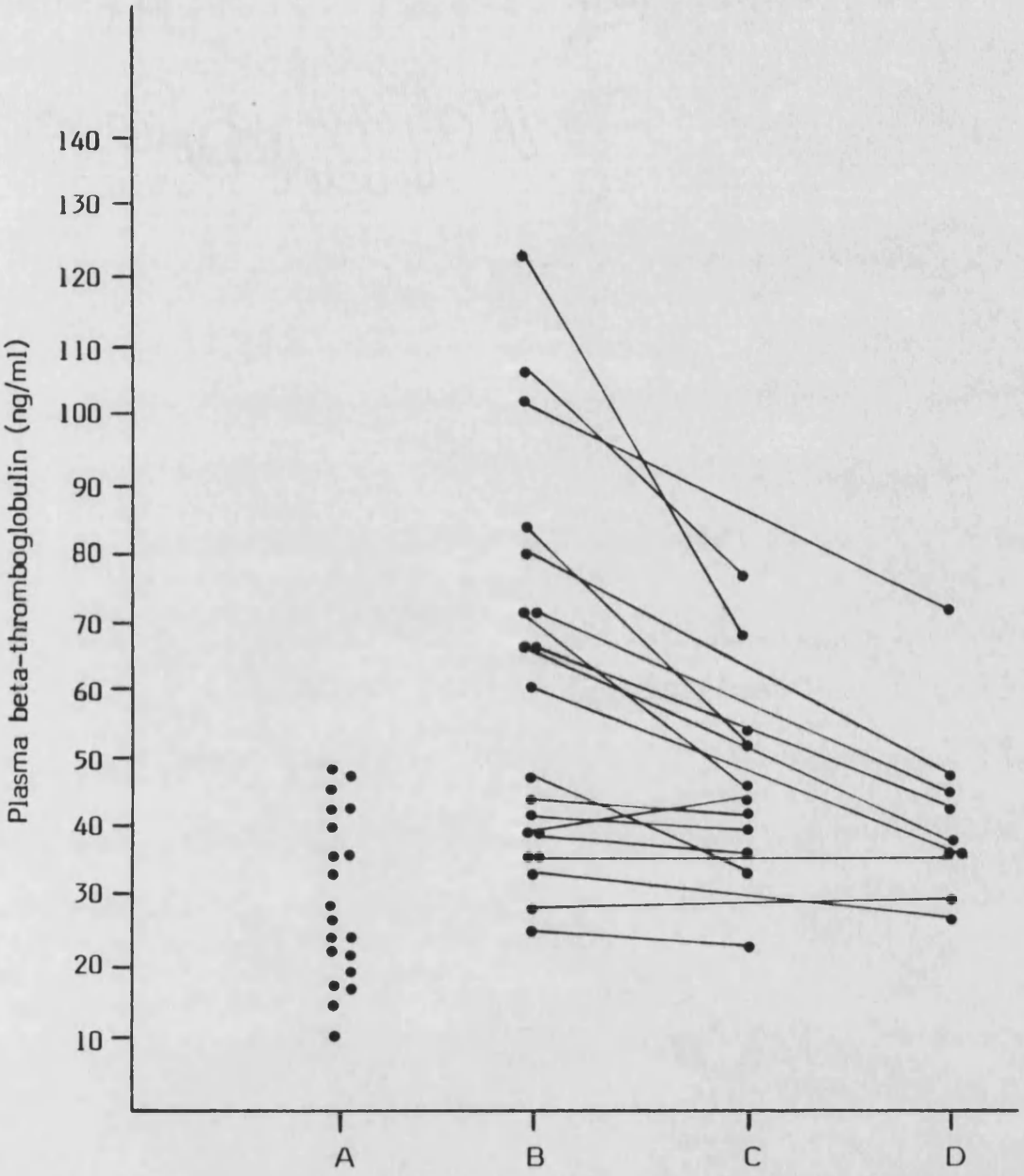


Fig. 4.3.b Plasma beta-thromboglobulin levels in (A) Normal Controls, (B) R.A. Patients Pre D-pen treatment week 0, (C) R.A. Patients D-pen Treated at 4 weeks, (D) R.A. Patients D-pen treated at 8 weeks. Statistical analysis shows:- a significant difference between A vs B ($P < 0.001$).

RA DISEASE ACTIVITY SCORE	BETA THROMBOGLOBULIN LEVELS (ng/ml)
1 (n=0)	-
2 (n=5)	59 ± 16
3 (n=4)	66 ± 32
4 (n=11)	62 ± 35
Total RA (n=20)	62 ± 30
Controls (n=20)	32 ± 12

Table 5 Plasma beta-thromboglobulin levels at various stages of disease activity in rheumatoid arthritis Pre D-pen treatment. The results are expressed as mean ± S.D.

The mean plasma concentration of B-TG in RA-NSAIDs patients with classical rheumatoid arthritis was found to be 63±30 ng/ml (range 28-128 ng/ml). This was significantly greater ($P < 0.001$)than the levels found in healthy normal controls mean 32±12 ng/ml (range 10-51 ng/ml) as shown in Table 4. Following treatment with D-pen 250/mg day for 1 month, and 375mg/day for 2 months, the B-TG levels were again determined in the rheumatoid group (Fig 4.3.b) The mean level of B-TG had fallen significantly to 47±16 ng/ml (range 24-82 ng/ml) ($P < 0.05$). Closer examination of the data revealed, that, before D-pen therapy 50% of the RA group had B-TG levels similar to the control group and 50% were higher (Fig.4.3.b). However, as shown in Table 5 this difference did not correlate with the severity of the disease, or with age, sex and duration of rheumatoid arthritis.

4.3 Platelet malonyldialdehyde formation (MDA): An indication of platelet hyperfunction

The formation of malonyldialdehyde, a metabolite of prostaglandin endoperoxides, has been studied in platelet rich plasmas in response to stimulation by ADP, adrenaline and collagen.

Platelet prostaglandin synthesis plays a key role in platelet aggregation and in the platelet release reaction. Therefore in view of the initial findings concerning platelet

aggregation and their responses to ADP, adrenaline and collagen within the rheumatoid groups, a study was made on platelet suspensions from these groups to see if malonyldialdehyde generation was either impaired or increased by drugs used in rheumatoid arthritis especially D-pen.

4.3.1. Total platelet MDA generation in response to ADP, adrenaline and collagen in controls, RA-NSAIDs treated, RA-pre-D-pen and RA-D-pen treated patients

Using an LKB Ultrospec U.V. spectrophotometer and a thiobarbituric acid method for total MDA production peaks were seen at 532 nanometers. The addition of the aggregating agents ADP, adrenaline and collagen to the platelet rich plasma in the aggregometer induced the synthesis of MDA from platelets, whereas no increase in absorbance at 532 nanometers (<0.02 OD nmol/10^9) was observed when phosphate buffered saline was added to the platelet rich plasma in the test system.

Preliminary experiments with lower concentration of stimulating agents ADP 1.5-5 μmol/l, adrenaline 5-10 μmol/l, and collagen 1-3 μg/ml, induced only small but variable amounts of MDA in normal subjects. However, on increasing the concentrations to 10 μmol/l ADP, 20 μmol/l adrenaline and

4 μg/ml collagen stable and reproducible levels of MDA production were obtained. Table 6 illustrates that although total MDA production by RA-platelets pre-D-pen was decreased with all three agonists, they did not vary significantly between patients showing marked differences in RA disease activity.

Results of platelet MDA formation measured at 532 nanometers in the presence of ADP 10μmol/l are depicted in Fig.4.4.a. The normal control group who manifested normal platelet function without evidence for platelet hyperaggregability, generated values of platelet MDA mean 0.182±0.017 nmol/10^9 platelets. This was highly significantly different to the decreased values of MDA produced by RA-NSAIDs group mean 0.092±0.035 nmol/10^9 platelets and RA-D-pen group mean 0.080±0.021 nmol/10^9 platelets ($P < 0.001$), ($P < 0.001$) respectively.

Fig.4.4.b shows that NSAIDs, pre-D-pen and post-D-pen groups all produced significant decreases of total levels of MDA generation compared with healthy controls when induced by collagen 4 μg/ml. MDA production for NSAIDs mean 0.071±0.035 nmol/10^9 platelets, pre-D-pen mean 0.049±0.029 nmol/10^9 platelets and post D-pen mean 0.051±0.021 nmol/10^9 platelets versus normal control mean 0.085±0.027 nmol/10^9 platelets ($P < 0.001$).

As illustrated in Fig.4.4.c the generation of MDA in response

RA DISEASE ACTIVITY SCORE		MALONYLDIALDEHYDE PRODUCTION ($n/mol/10^9$ PLATELETS STIMULATED BY		
		ADP 10.0µmol/l	ADRENALINE 20.0µmol/l	COLLAGEN 4.0µg/ml
1	(n=0)	-	-	-
2	(n=5)	.102 ± .029	.070 ± .039	.046 ± .028
3	(n=4)	.088 ± .027	.075 ± .033	.055 ± .033
4	(n=11)	.095 ± .030	.067 ± .032	.047 ± .025
Total RA	(n=20)	.096 ± .029	.072 ± .036	.049 ± .029
Controls	(n=20)	.182 ± .017	.166 ± .022	.085 ± .027

TABLE 6 MDA levels at various stages of disease activity Pre D-pen treatment. The results are expressed as mean ± S.D.

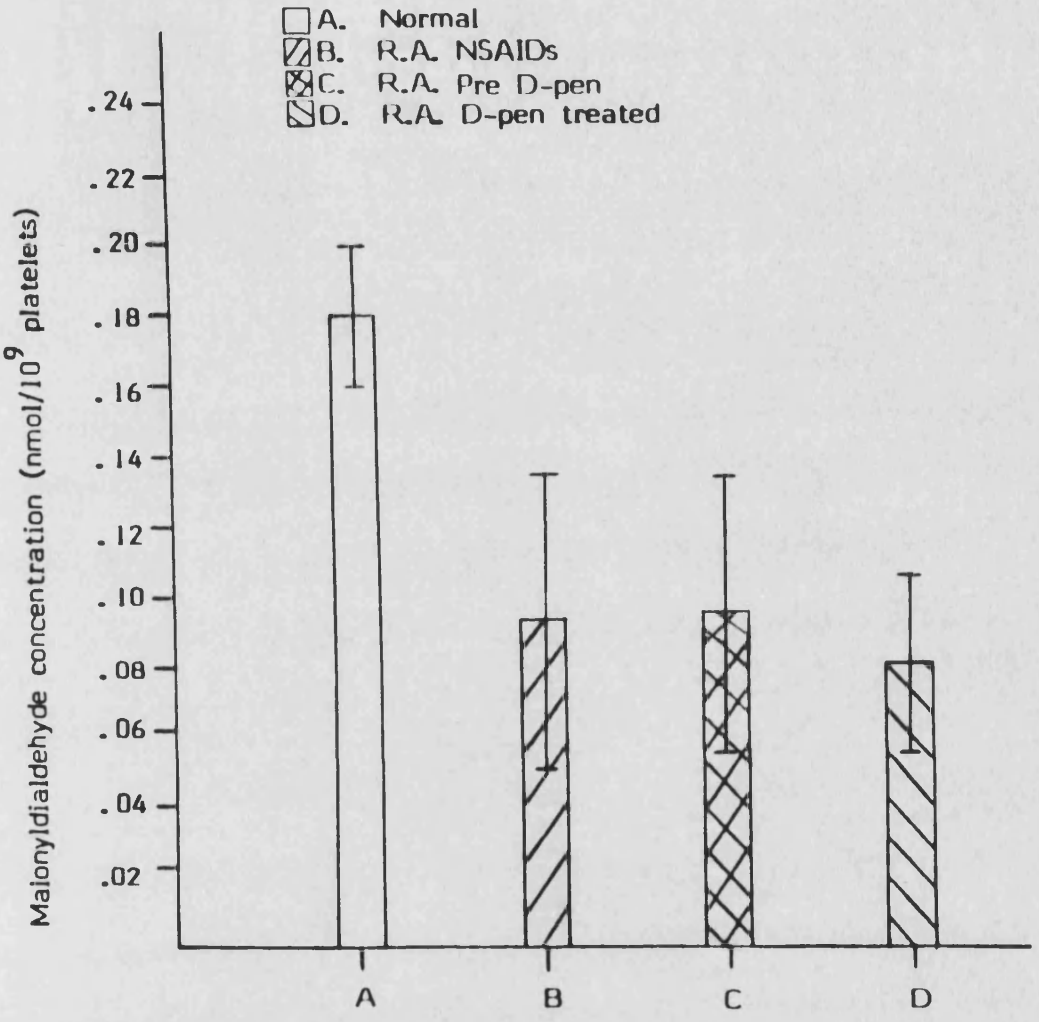


Fig. 4.4.a The effects of ADP 10μmol/l on Malonyldialdehyde generation from platelets (nmol/10^9 platelets of (A) Normal control, (B) R.A. - NSAIDs treated, (C) R.A. Pre D-pen (0 months), (D) R.A. - D-pen treated (3 months). The results are expressed as means ± S.D. (n = 20). Statistical analysis shows:- a highly significant difference between A vs B, C & D ($P < 0.001$).

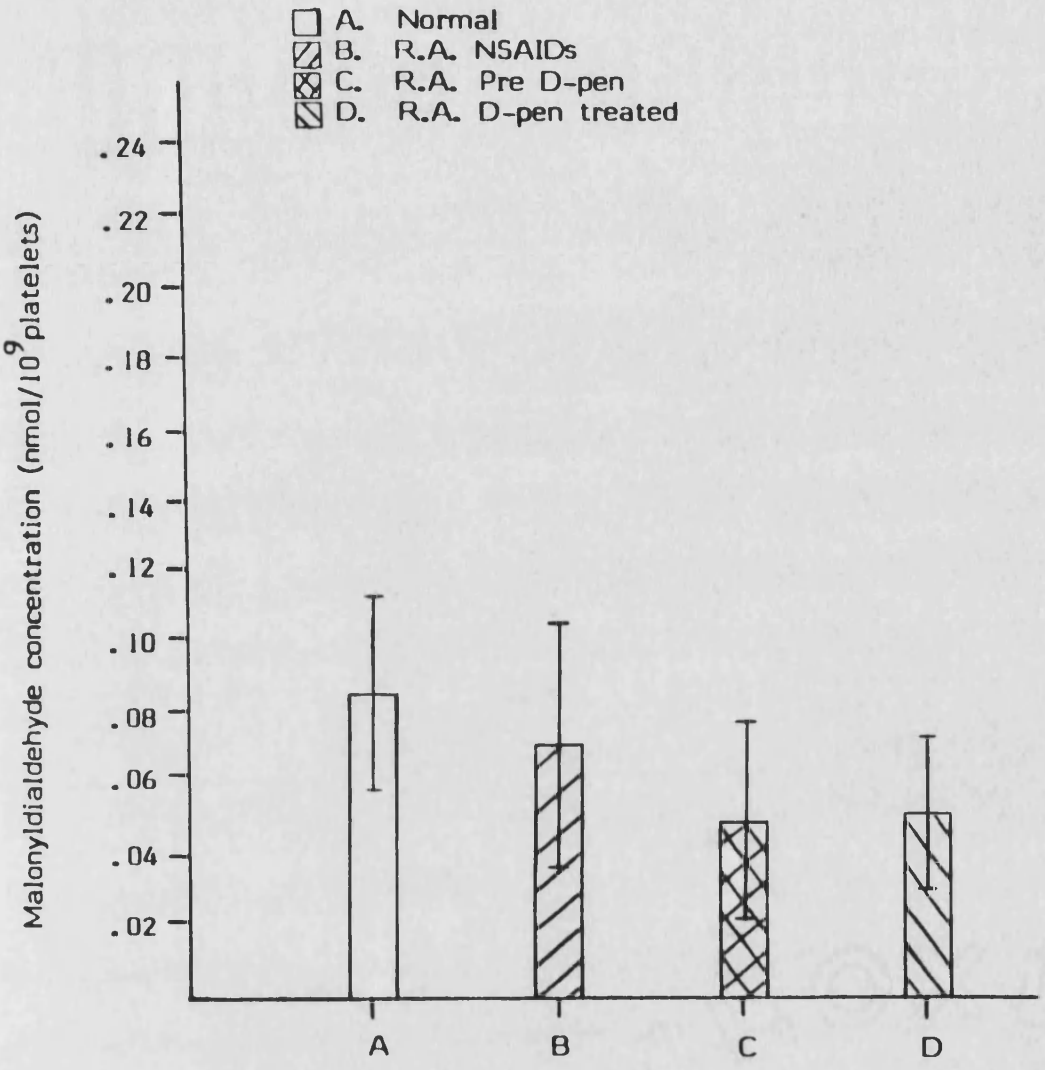


Fig. 4.4.b The effects of Collagen 4µg/ml on Malonyldialdehyde generation from platelets (nmol/10^9 platelets of (A) Normal control, (B) R.A. - NSAIDs treated, (C) R.A.Pre D-pen (0 months), (D) R.A. - D-pen treated (3 months). The results are expressed as means ± S.D. (n = 20). Statistical analysis shows:- a highly significant difference between A vs C and D ($P < 0.001$).

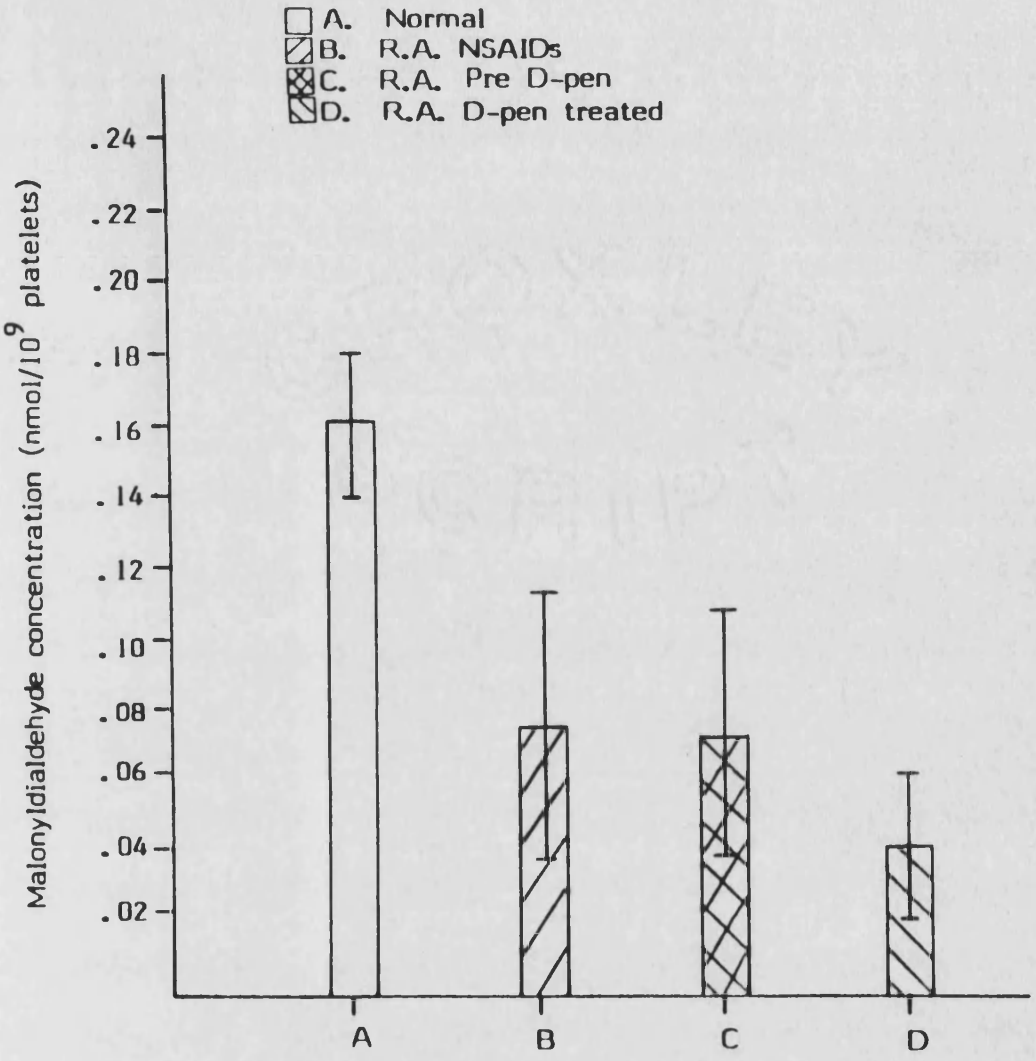


Fig. 4.4.c The effects of Adrenaline 20μmol/l on Malonyldialdehyde generation from platelets (nmol/10^9 platelets of (A) Normal control, (B) R.A. - NSAIDs treated, (C) R.A. Pre D-pen (0 months), (D) R.A. - D-pen treated (3 months). The results are expressed as means ± S.D. (n = 20). Statisical analysis shows:- a highly significant difference between A vs B, C & D ($P < 0.001$).

to adrenaline from platelets of RA-D-pen treated group were markedly depressed after 3 months mean 0.038±0.019 nmol/10^9 platelets, compared with MDA production at 0 months pre-D-pen mean 0.072±0.036 nmol/10^9 platelets ($P < 0.001$). There was also a significant difference in MDA levels between controls mean 0.160±0.022 nmol/10^9 platelets and RA-NSAIDs mean 0.074±0.037 nmol/10^9 platelets ($P < 0.001$). These data parallel observations made in platelet aggregation experiments stimulated with adrenaline. Here, the platelet response to adrenaline had decreased rapidly over 1 - 3 months with almost total loss of sensitivity to the agonist (see Fig.4.5.b).

To be sure that only MDA produced by the cyclooxygenase pathway of arachidonic acid was measured and not any thiobarbituric acid reactive substance produced by the lipoxygenase pathway of arachidonic acid, acetylsalicylic acid (1mM) inhibition experiments were also performed according to the method of Takayama, Okuma & Uchino, (1980) (data not shown). Complete inhibition of the generation of thiobarbituric acid reactive material was found in ADP, adrenaline and collagen stimulated samples from all patients studied.

4.4 Platelet aggregation

Serial studies were performed on three groups of patients,

twenty healthy controls, twenty RA-NSAID-treated and twenty patients before and after treatment with D-pen. Platelet rich suspensions were examined at monthly intervals for platelet aggregation as previously described in Chapter 2 (B) 2.1 and 2.2 and their responses to ADP, adrenaline and collagen recorded and plotted over a three month period.

4.4.1 Effects of D-pen treatment (0 - 3 months) on ADP, adrenaline and collagen induced platelet aggregation

As illustrated in Table 7, the various stages of disease activity pre-treatment had no significant effect on platelet aggregation responses. The result of the responses of patient groups to ADP are illustrated in Fig.4.5.a. The data shown were obtained with ADP concentrations equivalent to the ED_{50} of normal platelet responses. Healthy normal controls remained fairly constant over the time course averaging approximately 50% aggregation on each visit.

The RA NSAIDs group also showed very little change over the three months, but overall showed an increased sensitivity to ADP. However, in the RA group receiving D-pen aggregation responses decreased from an initial level at month 0 of 88% to 72% at month 1 and 68% at month 3 thereby indicating a significant loss of sensitivity to ADP.($P < 0.001$).

As illustrated in Fig.4.5.b the responses to adrenaline of platelets from RA-D-pen patients showed a dramatic inhibition of platelet aggregation, falling from a response at month 0 of 78% to 12% at month 1 and 5% at month 3 ($P < 0.001$). When viewing the data of platelets from RA NSAIDs treated and normals there was little variation over the 3 month period and only a minimal loss of sensitivity in the RA-NSAIDs was noted. Again the RA-NSAIDs showed an increased sensitivity to adrenaline at time 0. There was no apparent change in the control group. Several of these experiments were repeated after 18 months D-pen (data not shown) and the unresponsiveness of RA platelets to increasing concentrations of adrenaline remained.

As shown in Fig.4.5.c platelet aggregation stimulated with collagen 2.5 µg/ml shows a significant change over the period 0 - 3 months between the normal and RA-D-pen group with a progressive loss of sensitivity to collagen. The mean platelet aggregation in the RA-D-pen group performed at month 0 was 95%, and at 3 months the mean platelet aggregation had decreased to 63% almost the level of the normal controls. This is highly significant ($P < 0.001$) The control group remained fairly consistent over this time period, whilst the RA-NSAIDs showed a minimal loss of sensitivity to the collagen, mean level 94% month 0, mean level 88% month 3.

DISEASE ACTIVITY RA SCORE	PLATELET AGGREGATION ED_{50}		
	ADP µmol/l	ADRENALINE µmol/l	COLLAGEN µg/ml
1 (n=0)	-	-	-
2 (n=5)	0.74 ± .30	1.70 ± 1.07	1.08 ± .49
3 (n=4)	0.55 ± .19	1.87 ± 1.23	0.80 ± .16
4 (n=11)	0.50 ± .21	1.99 ± 1.02	1.07 ± .33
Total RA (n=20)	0.57 ± .24	1.90 ± 1.02	1.02 ± .35
Controls (n=20)	0.93 ± .38	2.48 ± 1.48	3.38 ± .99

Table 7 ADP, Adrenaline and Collagen ED_{50} concentration required for Platelet Aggregation at various stages of Disease Activity. The results are expressed as means ± S.D.

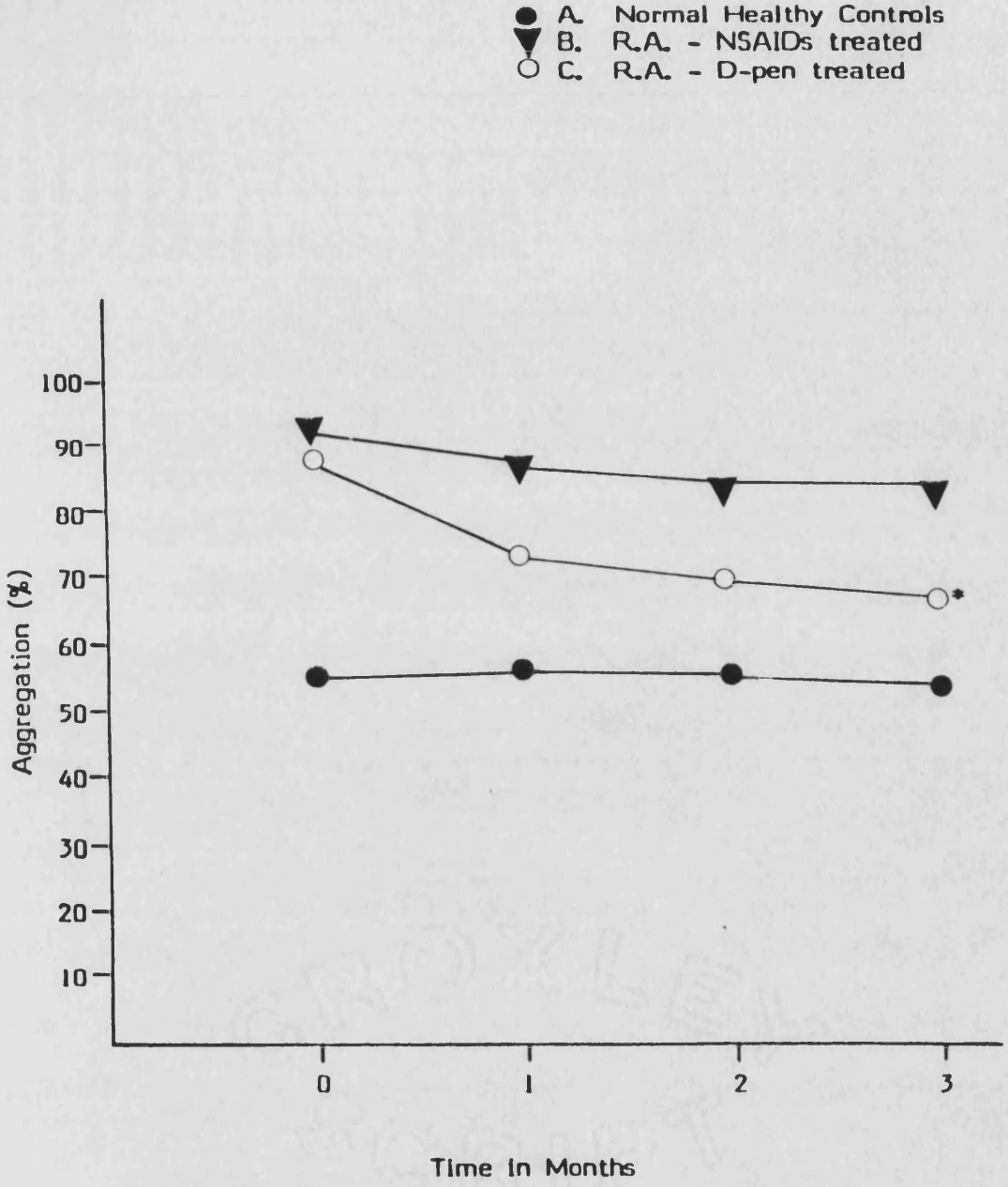


Fig.4.5.a Serial studies showing the effects of ED_{50} ADP 0.8μmol/l on platelet aggregation (%) in (A) Normal Healthy Controls, (B) R.A. NSAIDs treated, (C) R.A. D-pen treated patients (0 - 3 months). The results are expressed as mean ± S.D. (n = 20). The S.D. bars are omitted for clarity. Statistical analysis shows:- a highly significant difference between Month 0 vs Month 3 R.A. D-pen* ($P < 0.001$).

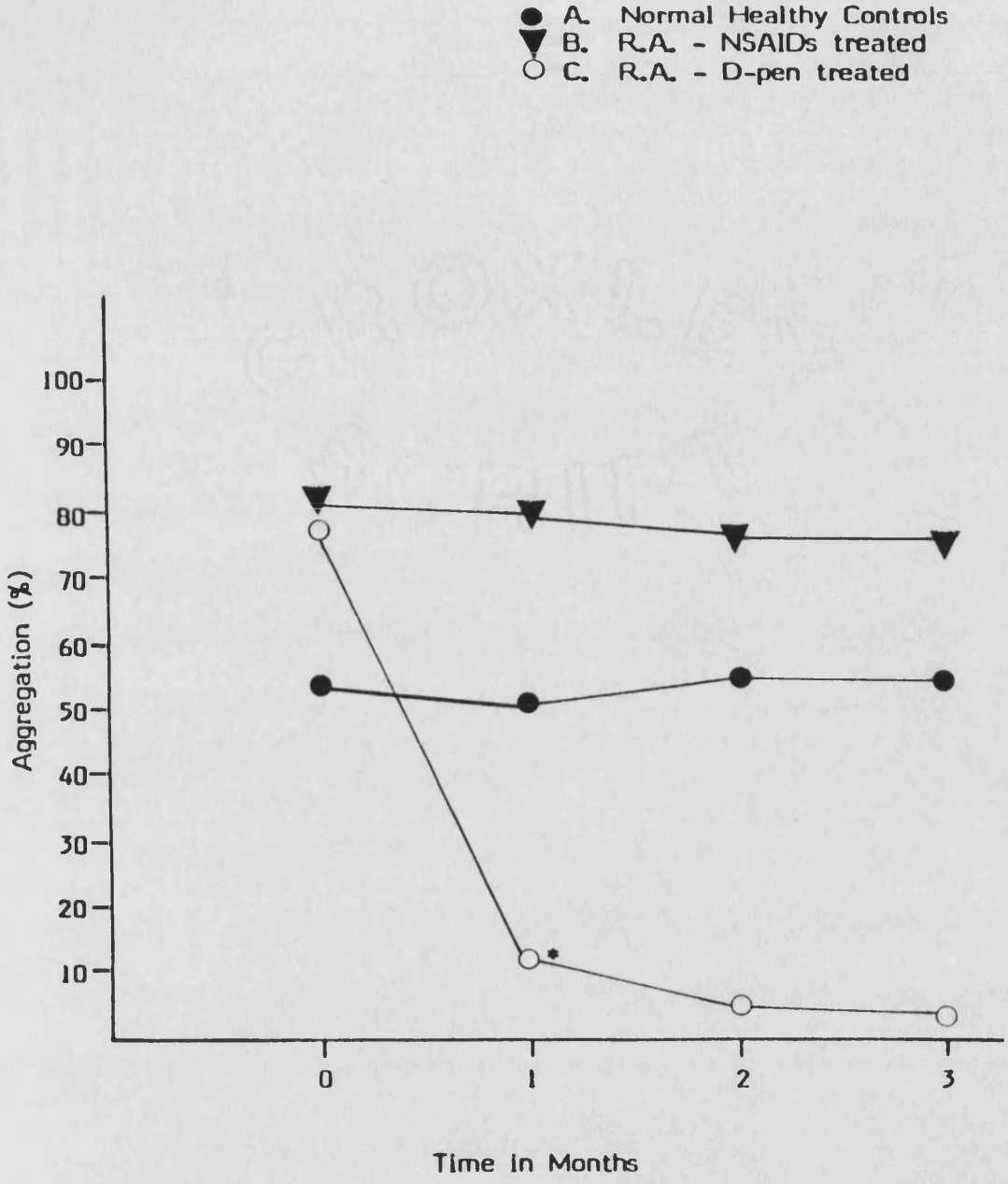


Fig.4.5.b Serial studies showing the effects of ED_{50} Adrenaline 2.0μmol/l on platelet aggregation (%) in (A) Normal Healthy Controls, (B) R.A. NSAIDs treated, (C) R.A. D-pen treated patients (0 - 3 months). The results are expressed as mean $\pm$ S.D. (n = 20). The S.D. bars are omitted for clarity. Statistical analysis shows:- a highly significant difference between Month 0 vs Month 1 R.A. D-pen* ($P < 0.001$).

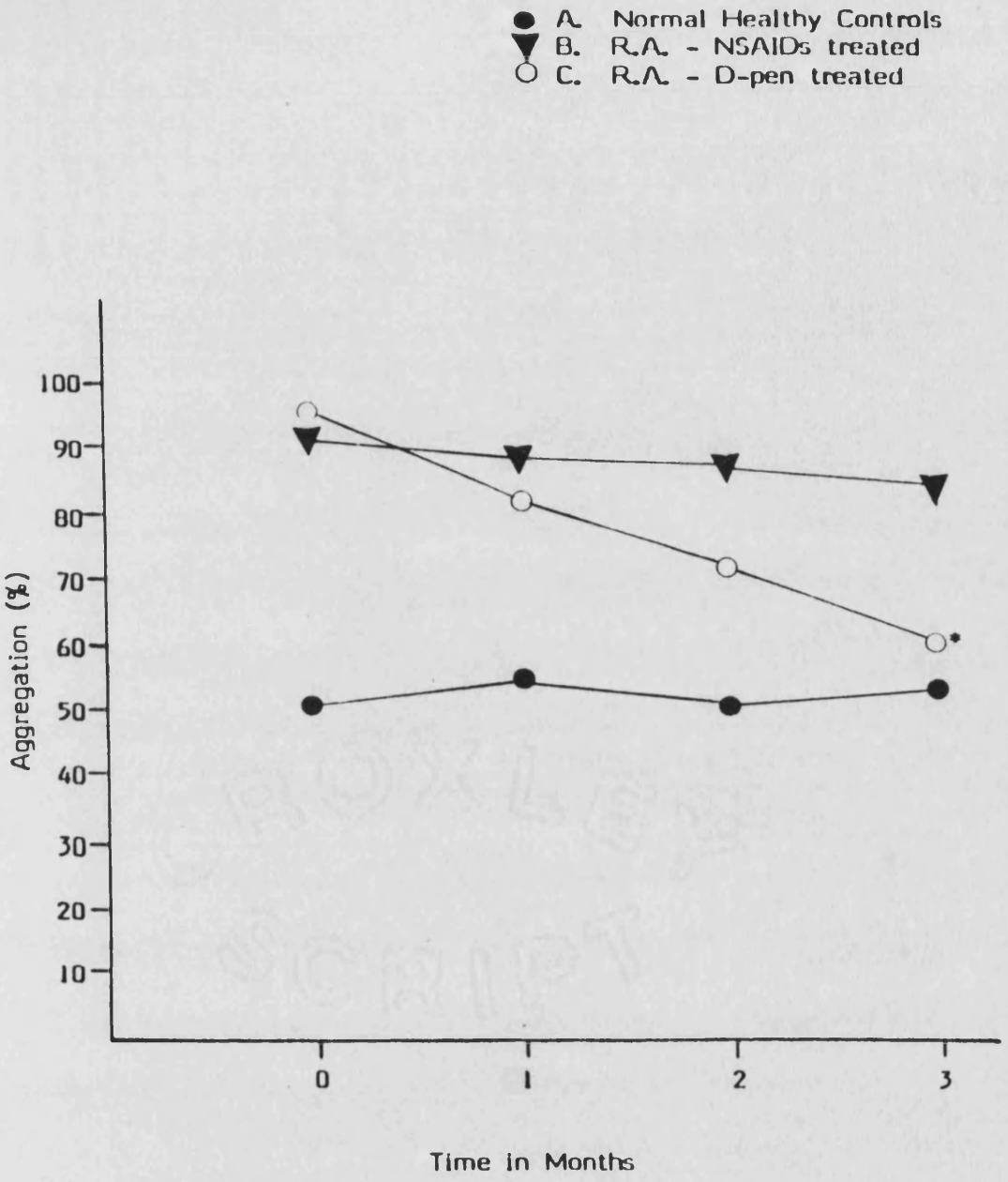


Fig.4.5.c Serial studies showing the effects of ED_{50} Collagen 2.5µg/ml on platelet aggregation (%) in (A) Normal Healthy Controls, (B) R.A. NSAIDs treated, (C) R.A. D-pen treated patients (0 - 3 months). The results are expressed as mean ± S.D. (n = 20). The S.D. bars are omitted for clarity. Statistical analysis shows:- a highly significant difference between Month 0 vs Month 3 R.A. D-pen* ($P < 0.001$).

4.5 Effects of D-Pen treatment on B-TG, MDA and platelet aggregation in rheumatoid patients showing good or poor clinical responses

Table 8 illustrates the changes in Beta-thromboglobulin levels, MDA production and ED_{50}'s for platelet aggregation indicating a good or poor clinical response. Levels of Beta-thromboglobulin in the responder group showed a significant decrease in total B-TG mean 43 ± 13 ng/ml compared to the non responders mean 65 ± 12 ng/ml ($P < 0.05$).

With MDA production there was no significant difference between the good or poor clinical response groups upon stimulation with ADP 10.0 µmol/l, collagen 4.0 µg/ml and adrenaline 20.0 µmol/l.

With ED_{50} aggregation the responder group showed a slight increase in sensitivity to lower concentrations of ADP, mean 0.69 ± 0.28 µmol/l, compared to a non responder mean 0.75 ± 0.17 µmol/l. Adrenaline responses were absent both in the responder and non responder groups, whilst collagen showed a slight increase in sensitivity within the non responder group mean 1.40 ± 0.37 µg/ml compared to responder group mean 1.58 ± 0.35 µg/ml. However, statistical analysis of these results proved to be non significant.

	RESPONDERS (n=16)	NON RESPONDERS (n=4)
B-Thromboglobulin (ng/ml)	43 ± 14	65 ± 12*
MDA Production ($nmol/10^9$ plts) stimulated by		
ADP 10.0µmol/l	.079 ± .020	.083 ± .029
Adrenaline 20.0µmol/l	.036 ± .023	.045 ± .023
Collagen 4.0µg/ml	.051 ± .021	.052 ± .023
ED_{50} for Platelet Aggregation		
ADP µmol/l	0.69 ± .28	0.75 ± .17
Adrenaline µmol/l	>100.00	>100.00
Collagen µg/ml	1.58 ± .35	1.40 ± .37
Mean dose of D-pen mg/day	406 ± 97	593 ± 120

TABLE 8 Levels of Beta-thromboglobulin, MDA and ED_{50} platelet aggregation in D-pen treated rheumatoid patients showing good or poor clinical responses. Results are expressed as mean ± S.D. Statistical analysis shows:- a significant difference between B-TG responder vs non-responder* ($P < 0.05$).

CHAPTER FIVE:

RESULTS - IN VITRO STUDIES

5.1 Human platelet alpha$_2$ adrenoceptors

Adrenaline, acting via an alpha$_2$ adrenergic receptor, stimulates platelets to aggregate and secrete, and potentiates the aggregation and secretion induced by unrelated agonists. Therefore platelets may be useful for studying changes in alpha$_2$ adrenergic receptor numbers or functions in human diseases and in defining molecular events involved in alpha adrenergic actions. These receptors have been characterised by radioligand binding techniques using (^{3}H) dihydroergocryptine (Boullin & Elliott, 1979); (^{3}H) clonidine (Shattil, McDonough, Turnbull & Insell, 1981); and by direct binding to intact human platelets of (^{3}H) yohimbine (Boon, Elliott, Grahaeme-Smith, Outlaw & Stump, 1981).

The aim of this investigation was to examine alpha$_2$ receptor expression on blood platelets from RA patients treated with D-pen in view of the loss of aggregation to adrenaline seen in these cells in earlier experiments.

5.1.1 (^{3}H) yohimbine binding assay for alpha$_2$ adrenergic receptors on normal, RA-NSAIDs, RA-D-pen treated platelets

Non specific binding was estimated by the addition of an excess (50μM) of unlabelled phentolamine to one set of assay tubes containing incubation medium and dilutions of (^{3}H)

yohimbine. Specific binding was taken to be the total amount of (^{3}H) yohimbine bound, minus the non specific binding in the presence of phentolamine after incubation at 37^{o}C for twenty minutes.

5.1.2 Dissociation constant (K_D) of alpha$_2$ adrenergic receptors on platelets in normal, RA-NSAIDs and RA-D-pen treated groups

Figs.5.1.a, and 5.1.b show the results of various concentrations of (^{3}H) yohimbine binding to normal intact platelets, RA-NSAIDs treated platelets and RA-D-pen treated platelets. Scatchard plots were derived from these data.

(^{3}H) yohimbine bound in a saturable manner to the intact platelets, and the Scatchard analysis indicated a single site. Non specific binding was 35 -50% of total binding at all concentrations of (^{3}H) yohimbine tested. Fig.5.1.e illustrates plots for affinity of the platelet receptors (K_D) for the radioligand (^{3}H) yohimbine in the three groups, control (K_D) mean 2.6±1.0 nmol/l, RA-NSAIDs mean 2.5±1.00 nmol/l and RA-D-pen mean 2.8±1.1 nmol/l. There was no significant difference in these (K_D) values, thereby indicating similar affinity for the receptor antagonist in the three groups studied.

5.1.3 Scatchard plots of data showing binding sites per platelet of normals, RA-NSAIDs and RA-D-pen patients

In six separate experiments the saturation binding isotherm of (^{3}H) yohimbine was compared with platelets from healthy controls, RA patients receiving NSAIDs and RA patients on D-pen. The maximum number of alpha$_2$ receptor binding sites (B_{max}) on the basis of (^{3}H) yohimbine specific binding on intact human platelets was determined by Scatchard anaylsis as shown in Fig.5.1.c and Fig.5.1.d. The results in Fig.5.1.f show that the mean number of binding sites for controls were 49.9±6.1 sites/platelet fmol/10^8, for RA-NSAIDs 47.2±8.9 fmol/10^8 cells and in RA-D-pen treated 35.2±6.8 fmol/10^8 cells. These results indicated that the number of alpha$_2$ receptor binding sites were significantly lower in RA-D-pen patients compared to normals, and indeed with RA-NSAIDs patients, ($P<0.01$, $P<0.05$) respectively.

Platelet aggregation studies were also performed with normal platelet rich plasma to demonstrate that the (^{3}H) yohimbine could antagonise the response to adrenaline. As much as 6μM yohimbine failed to aggregate platelets, but as little as 0.03μM inhibited the initial rate and extent of platelet aggregation induced by 10μM adrenaline. This inhibition was half maximal at 0.2μM and maximal at 1μM.

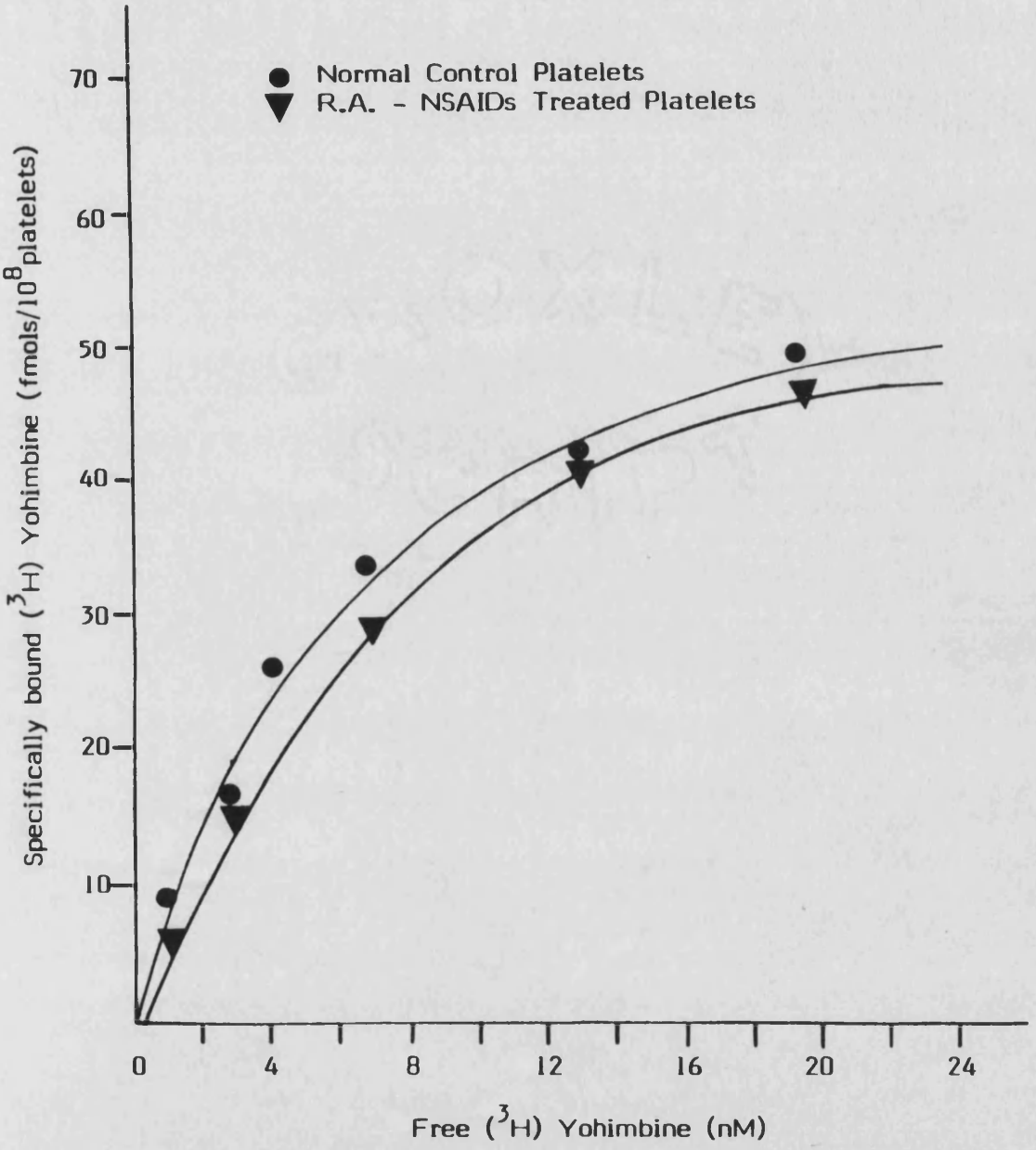


Fig.5.1.a Platelet Alpha$_2$ adrenoceptors. The equilibrium binding data of (^{3}H) Yohimbine for Normal and R.A. - NSAIDs platelets as a function of (^{3}H) Yohimbine concentration. One of six similar experiments.

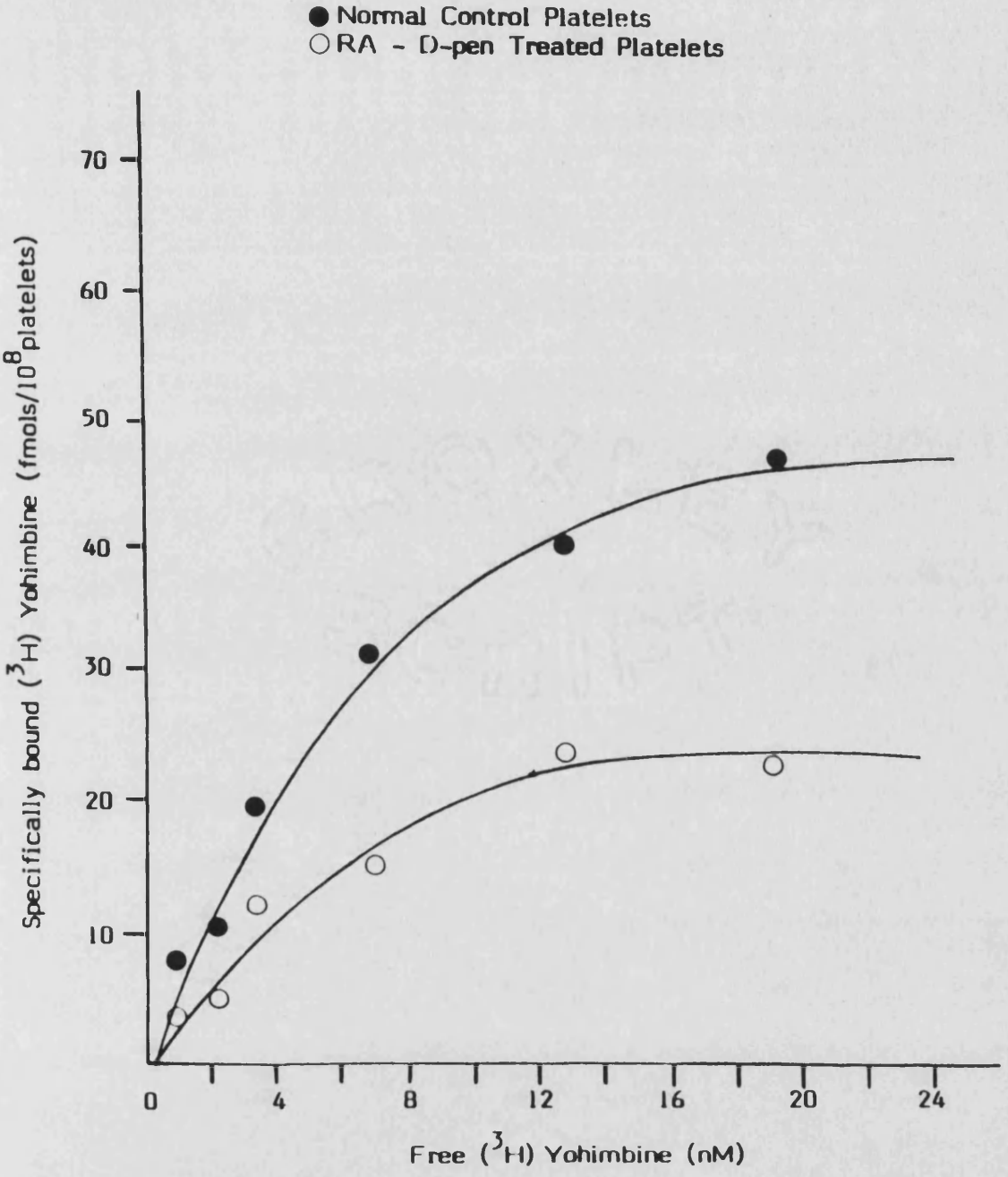


Fig. 5.1.b Platelet Alpha 2 adrenoceptors. The equilibrium binding data of (^{3}H) Yohimbine as a function of (^{3}H) Yohimbine concentration for normal controls and RA patients D-pen treated. One of six similar experiments.

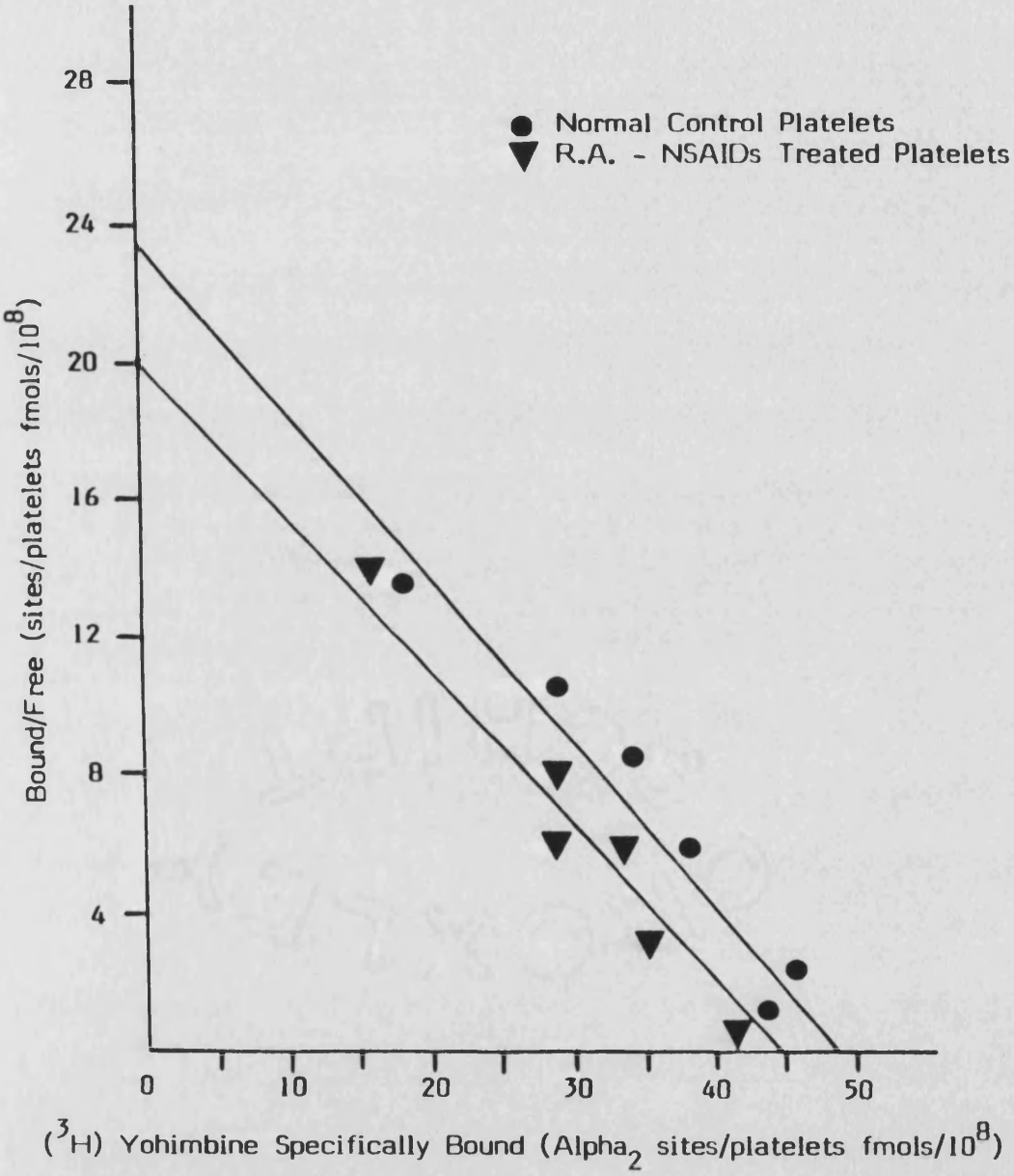


Fig. 5.1.c Scatchard plot of data showing binding sites per platelet of normal controls and R.A. - NSAIDs treated patients. Each value is the mean of triplicate determinations. One of six similar experiments.

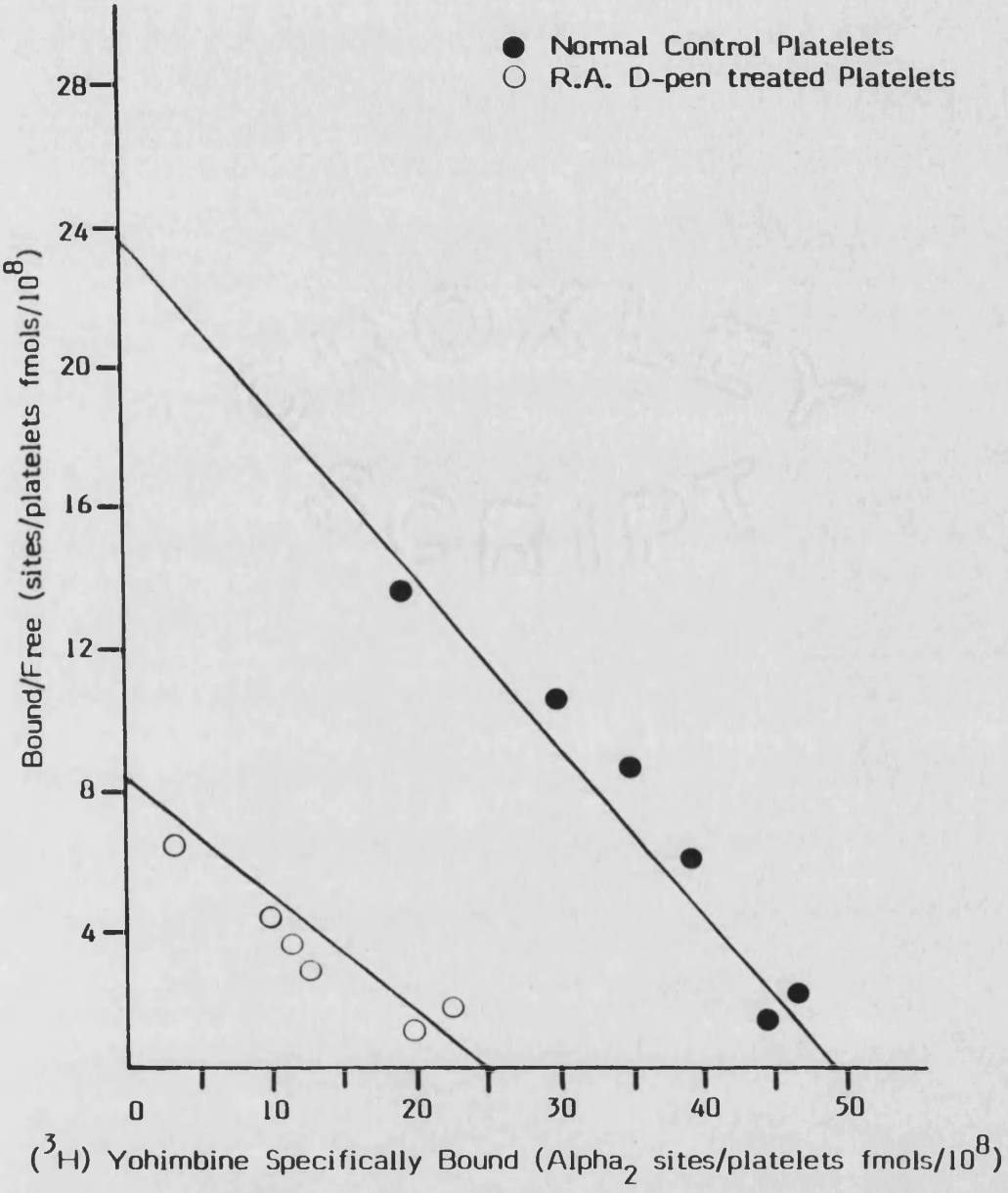


Fig. 5.1.d Scatchard plot of data showing binding sites per platelet of normal and R.A. D-pen treated patients. Each value is the mean of triplicate determinations. One of six similar experiments.

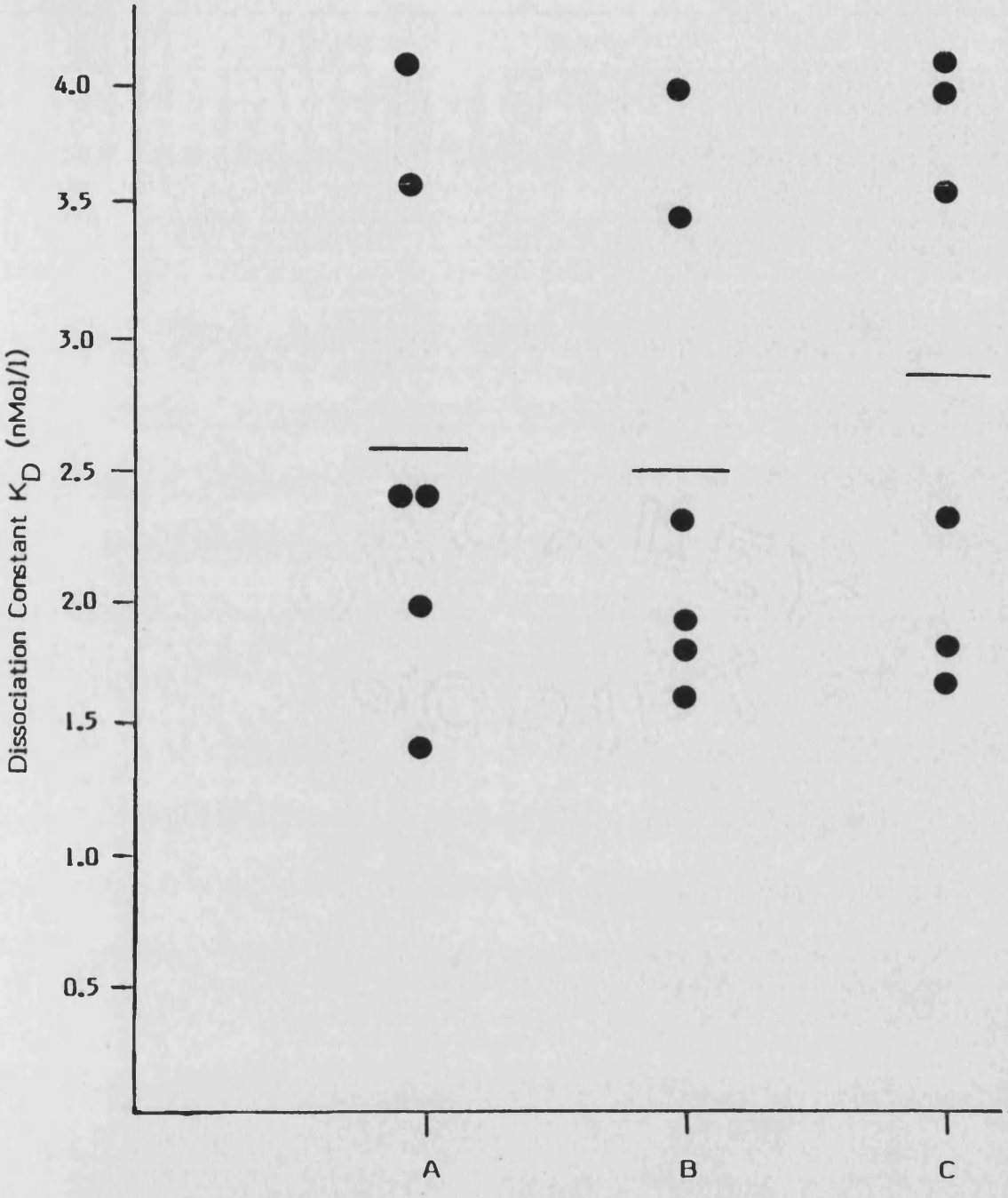


Fig. 5.1.e The dissociation constant of Alpha$_2$ adrenergic receptors on platelets in (A) Normal Controls, (B) R.A. - NSAIDs treated (C) R.A. - D-pen treated. Saturation binding was performed with (^{3}H) yohimbine binding to intact platelets and the dissociation constant (K_D) determined from Scatchard plots. The bars represent mean values (n = 6).

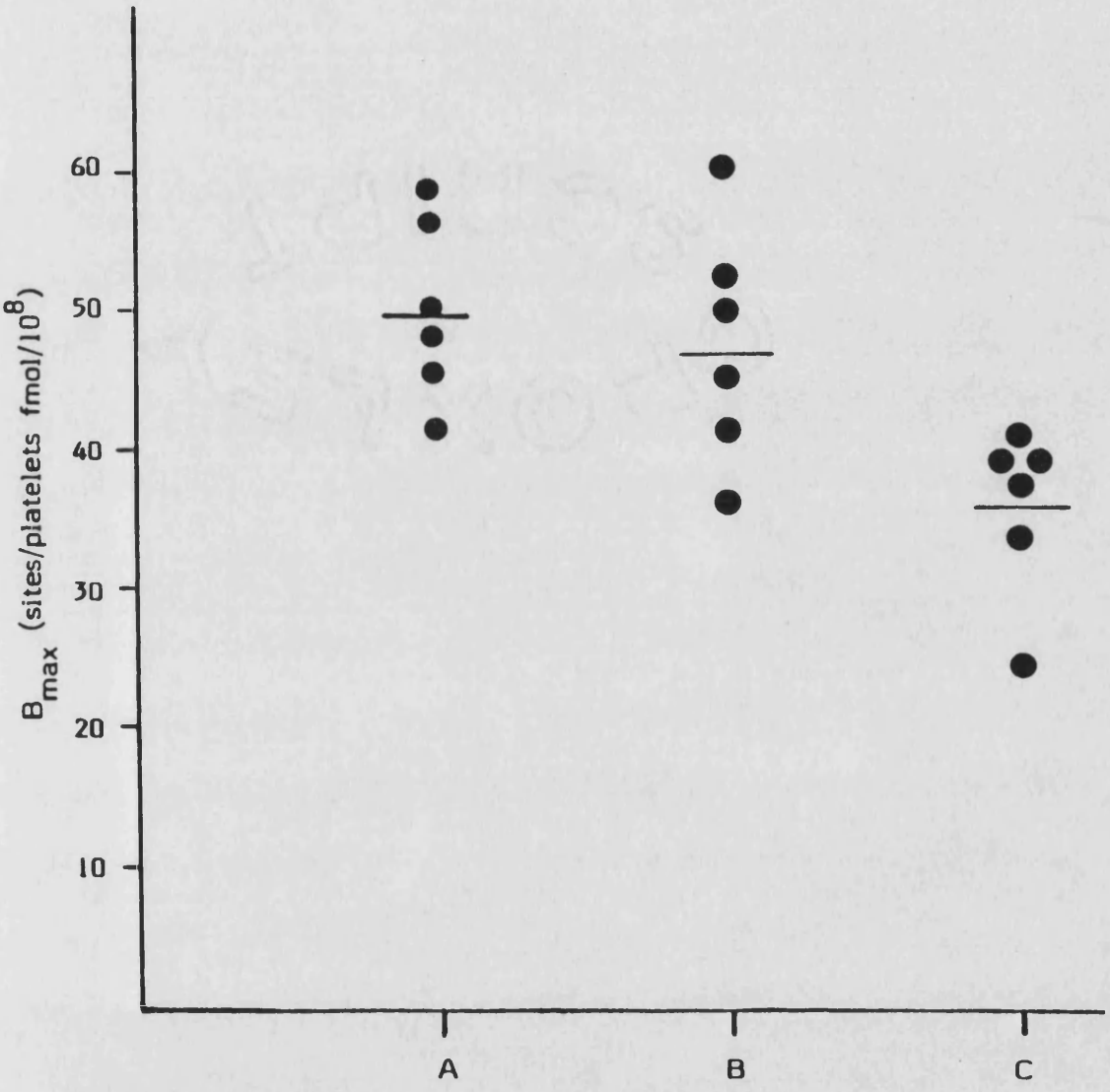


Fig. 5.1.f The number of Alpha$_2$ adrenergic receptors on platelets in (A) Normal Controls, (B) R.A. NSAIDs treated, (c) D-pen treated. Saturation binding was performed with (^{3}H) Yohimbine to intact platelets and the maximum binding (B_{max}) determined from Scatchard plots. The bars represent mean values (n = 6). Statistical analysis shows:- a significant difference between A vs C ($P < 0.01$) and B vs C ($P < 0.05$).

5.2 In vitro studies with D-pen, sodium aurothiomalate and other thiol reactive compounds

The observations described in vivo for D-pen show a marked effect of the drug on platelet function and in view of these findings attempts were made to reproduce these same observations in vitro using drugs or other chemicals known to react with sulphydryl groups. These included D-pen, sodium aurothiomalate, pHMPSA and cysteine which were used at various concentrations and incubation times in the following experiments.

5.2.1 The effects of in vitro incubation time and concentration of D-pen on platelet aggregation inhibition to ADP, adrenaline and collagen

In order to establish an optimum and maximum range for incubation times and concentrations of drugs used, preliminary experiments were performed with D-pen . The final range of incubation times selected were 10mins, 30mins and 60 mins, with concentrations of D-pen 5μg/ml, 10μg/ml, and 20μg/ml.

The results for in vitro D-pen are represented in Figs.5.2.a 5.2.b and 5.2.c the total platelet aggregation inhibition to ADP, adrenaline and collagen are shown in Table 9. The graphs show D-pen caused both time and drug concentration dependent

inhibition of platelet aggregation. There was also an apparent increase in spontaneous aggregation with time. In the test systems D-pen affected responses to all three platelet agonists.

The effect was most marked with adrenaline, followed by ADP and collagen. However, in this in vitro regime D-pen did not bring about such dramatic inhibition as in the in vivo experiments. Clearly, from Table 9 the inhibitory effect of D-pen on platelet aggregation was most marked with adrenaline, followed by ADP and finally collagen. This pattern was maintained at all incubation times and drug concentrations examined.

Statistical analysis of all the in vitro aggregation inhibition studies gave parallel results irrespective of incubation times or D-pen concentrations used. Examples of these were, adrenaline versus ADP at 5 minutes incubation and 5μg/ml D-pen ($P < 0.01$), ADP versus collagen ($P < 0.05$), adrenaline versus collagen ($P < 0.01$), with similar P values obtained at 30 minutes and 60 minutes incubation and at 10μg/ml and 20μg/ml concentrations of D-pen. The rank order noted with these agonists parallels the observed changes to platelet aggregation brought about by D-pen treatment in vivo.

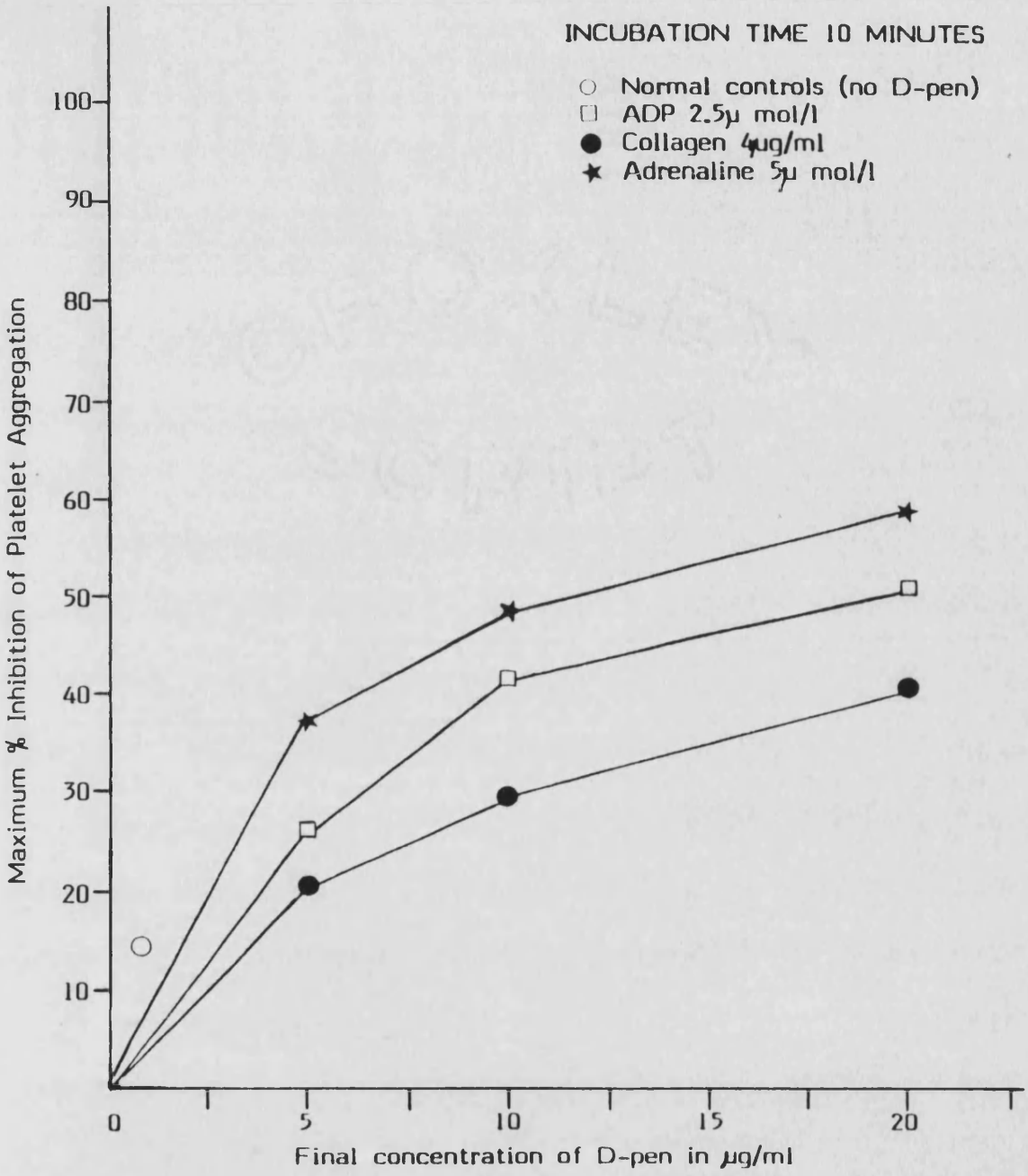


Fig.5.2.a The effects of in vitro incubation (37°C 10 mins) of various concentrations of D-penicillamine on % inhibition of platelet aggregation using agonists (1) ADP 2.5μ mol/l (2) Collagen 4ug/ml, (3) Adrenaline 5μ mol/l + controls (no D-penicillamine). The results are expressed as means (n = 10). The s.d. bars are omitted for clarity.

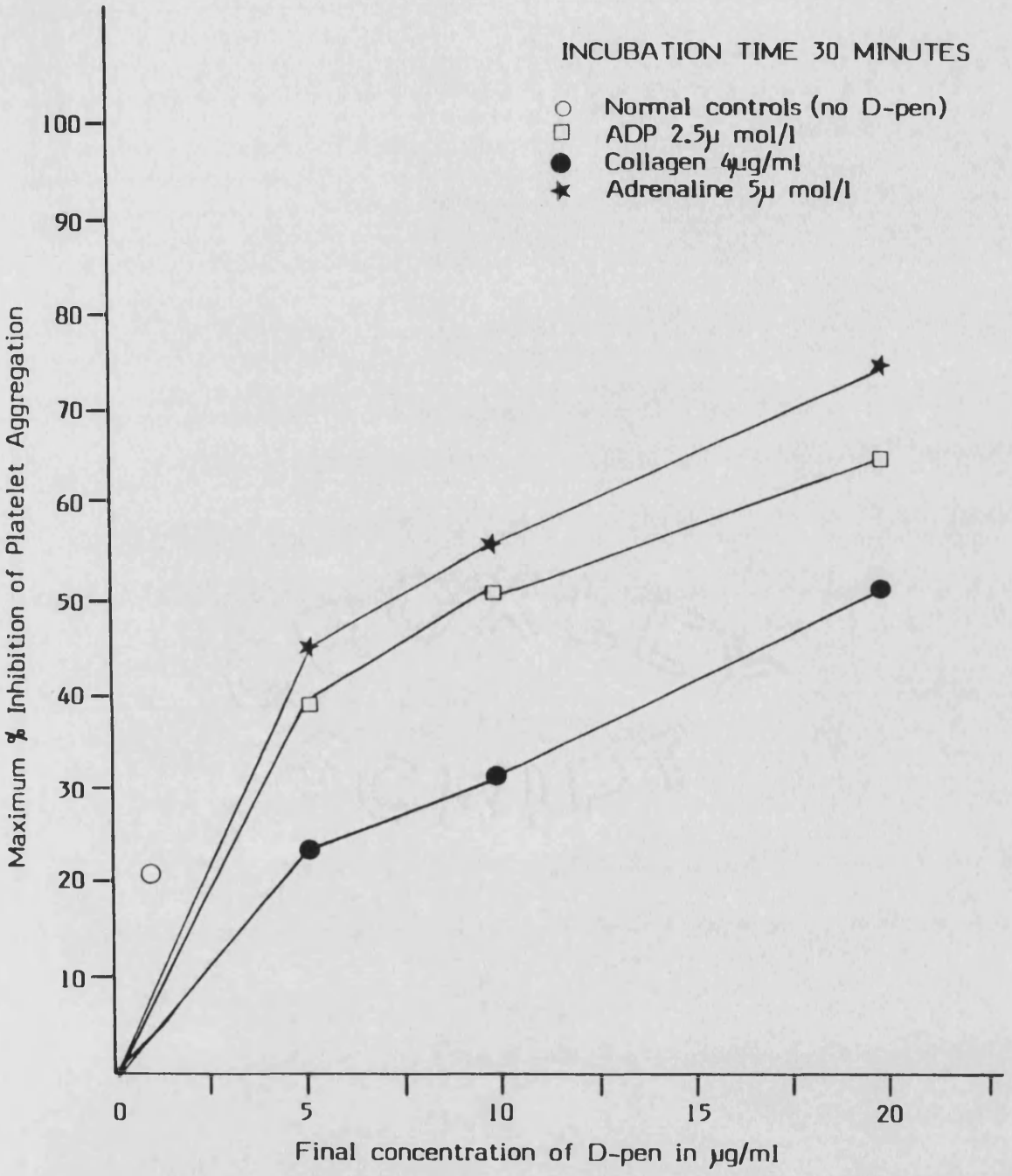


Fig. 5.2.b The effects of in vitro incubation (37°C 30 mins) of various concentrations of D-penicillamine on % inhibition of platelet aggregation using agonists (1) ADP 2.5μ mol/l (2) Collagen 4ug/ml, (3) Adrenaline 5μ mol/l + controls (no D-penicillamine). The results are expressed as means (n = 10). The s.d. bars are omitted for clarity.

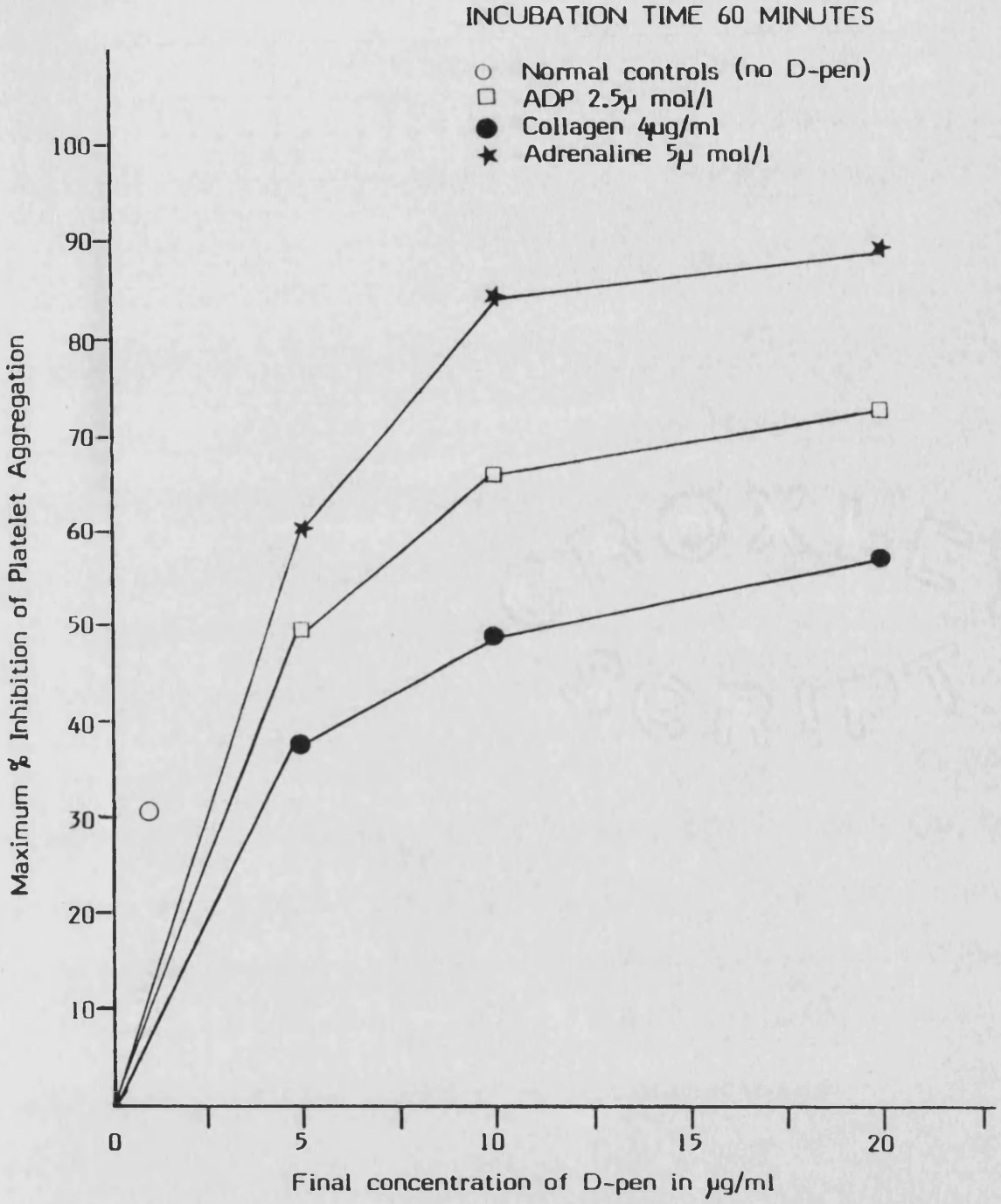


Fig. 5.2.c The effects of in vitro incubation (37°C 60 mins) of various concentrations of D-penicillamine on % inhibition of platelet aggregation using agonists (1) ADP 2.5μ mol/l (2) Collagen 4ug/ml, (3) Adrenaline 5μ mol/l+ controls (no D-penicillamine). The results are expressed as means (n = 10). The s.d. bars are omitted for clarity.

Pre Incubation Time	Final Concentration of D-pen	% Maximum Aggregation Inhibition Mean ± S.D. (n = 10)		
		A	B	C
	5µg/ml	26 ± 6	20 ± 6	38 ± 6
10 mins	10µg/ml	42 ± 7	28 ± 5	48 ± 6
	20µg/ml	51 ± 6	41 ± 7	60 ± 10
Normal Control	(No D-pen)	15 ± 4	12 ± 3	18 ± 6
	5µg/ml	39 ± 5	22 ± 4	45 ± 6
30 mins	10µg/ml	50 ± 7	31 ± 6	55 ± 9
	20µg/ml	63 ± 8	49 ± 7	75 ± 6
Normal Control	(No D-pen)	20 ± 6	19 ± 4	24 ± 6
	5µg/ml	50 ± 8	38 ± 8	60 ± 15
60 mins	10µg/ml	66 ± 7	49 ± 10	85 ± 7
	20µg/lm	73 ± 8	57 ± 9	90 ± 5
Normal Control	(No D-pen)	30 ± 7	28 ± 6	32 ± 8
Platelet Agonist		2.5µmol/l ADP	4µg/ml Collagen	5µmol/l Adrenaline

TABLE 9 Comparing the Inhibition of platelet aggregation by Pre Incubation of D-penicillamine 10, 30 and 60 minutes at various concentrations versus platelet agonists ADP 2.5µmol/l, Collagen 4µg/ml and Adrenaline 5µmol/l. The results are expressed as means ± S.D. Statistical analysis shows:- a significant difference between A vs C ($P < 0.01$); A vs B ($P < 0.05$) and B vs C ($P < 0.01$), (see text).

5.2.2 The effects of in vitro incubation time and concentration of Sodium aurothiomalate on platelet aggregation inhibition to ADP, adrenaline and collagen

A variety of enzyme systems are influenced by gold, particularly those requiring free SH groups. Comparisons of sodium aurothiomalate with D-pen (both sulphydryl compounds) have led to the intriguing proposal that they may act by a common mechanism. The observations found in in vivo and in vitro experiments with D-pen were extended to study sodium aurothiomalate in vitro and to compare these findings. The ability of sodium aurothiomalate to inhibit platelet aggregation was investigated at various incubation times and at different drug concentrations.

Preliminary experiments were performed in order to establish the optimal times and final drug concentration ranges needed to evaluate platelet aggregation inhibition with sodium aurothiomalate. Following these initial experiments incubation times selected were 10 mins, 30 mins, and 60 mins, and concentrations of sodium aurothiomalate 0.5μg/ml, 1.5μg/ml, 3μg/ml and 5μg/ml (final concentration).

The results shown in Figs 5.3.a, 5.3.b and 5.3.c illustrate that sodium aurothiomalate reacted in a similar manner to D-pen in vitro and caused both time and drug concentration

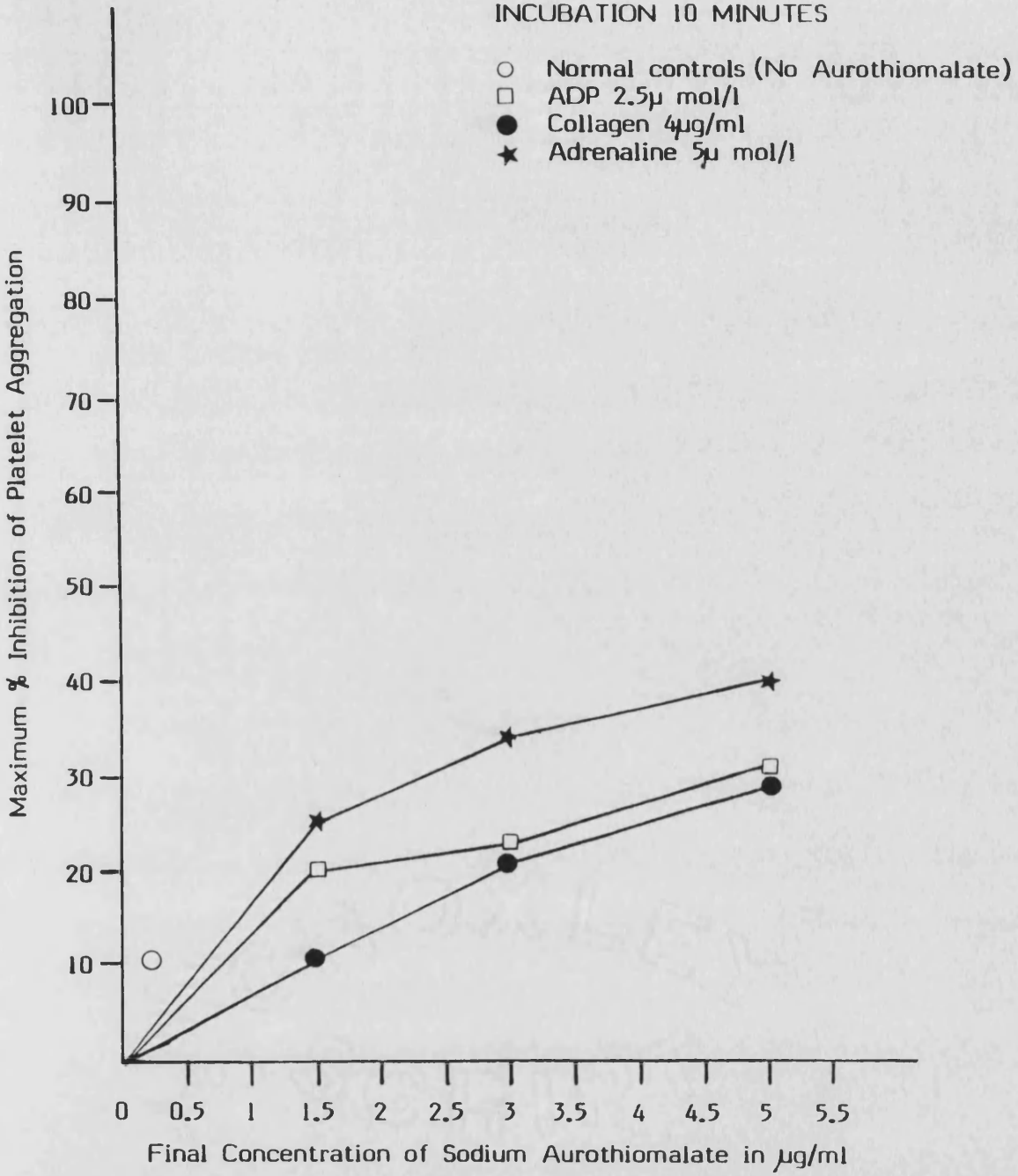


Fig. 5.3.a The effects of in vitro incubation (37°C 10 mins) of various concentrations of Sodium Aurothiomalate on % inhibition of platelet aggregation using agonists (1) ADP 2.5μ mol/l, (2) Collagen 4μg/ml, (3) Adrenaline 5μ mol/l+ controls (no Sodium Aurothiomalate). The results are expressed as means (n = 10). The s.d. bars are omitted for clarity.

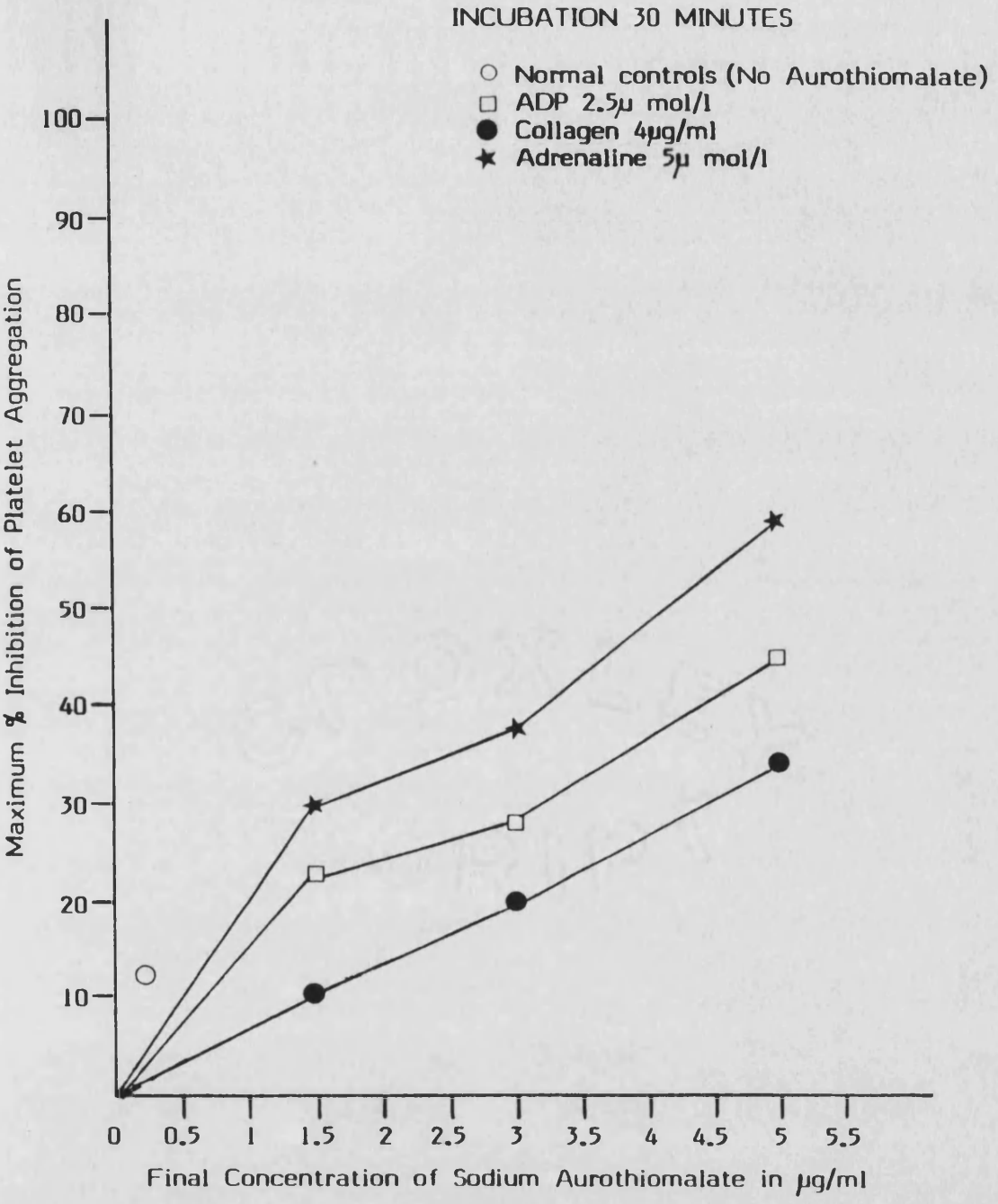


Fig.5.3.b The effects of in vitro incubation (37°C 30 mins) of various concentrations of Sodium Aurothiomalate on % inhibition of platelet aggregation using agonists (1) ADP 2.5μ mol/l, (2) Collagen 4μg/ml, (3) Adrenaline 5μ mol/l+ controls (no Sodium Aurothiomalate). The results are expressed as means (n = 10). The s.d. bars are omitted for clarity.

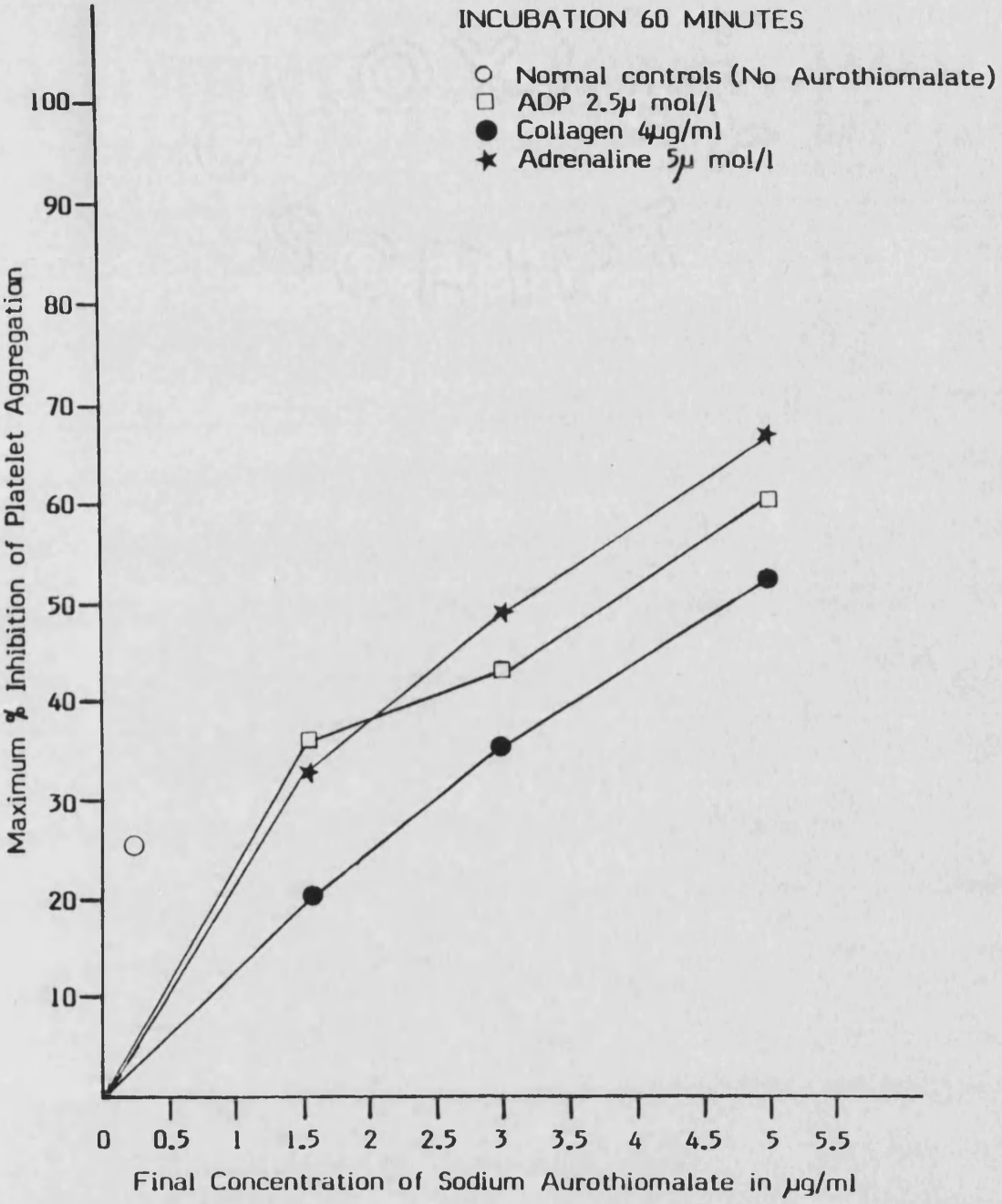


Fig.5.3.c The effects of in vitro incubation (37°C 60 mins) of various concentrations of Sodium Aurothiomalate on % inhibition of platelet aggregation using agonists (1) ADP 2.5μ mol/l, (2) Collagen 4μg/ml, (3) Adrenaline 5μ mol/l + controls (no Sodium Aurothiomalate). The results are expressed as means (n = 10). The s.d. bars are omitted for clarity.

Pre Incubation Time	Final Concentration of Aurothiomalate	% Maximum Aggregation Inhibition Mean ± S.D. (n = 10)		
10 mins	1.5µg/ml	20 ± 6	10 ± 3	24 ± 7
	3µg/ml	22 ± 7	20 ± 8	34 ± 5
	5µg/ml	30 ± 6	28 ± 6	40 ± 6
Normal Control	(No Aurothiomalate)	8 ± 3	10 ± 2	10 ± 4
30 mins	1.5µg/ml	22 ± 5	10 ± 4	30 ± 6
	3µg/ml	28 ± 5.5	20 ± 6	38 ± 7
	5µg/ml	46 ± 8	34 ± 8	59 ± 8
Normal Control	(No Aurothiomalate)	12 ± 3	10 ± 2	14 ± 4
60 mins	1.5µg/ml	35 ± 6	20 ± 5	33 ± 5
	3µg/ml	42 ± 8.5	35 ± 8	49 ± 6
	5µg/ml	60 ± 10.5	52 ± 9	67 ± 10
Normal Control	(No Aurothiomalate)	24 ± 6	20 ± 6	25 ± 5
Platelet Agonist		2.5µmol/l ADP	4µg/ml Collagen	5µmol/l Adrenaline

TABLE 10 Comparing the Inhibition of platelet aggregation by Pre Incubation of Aurothiomalate 10, 30 and 60 minutes at various concentrations versus platelet agonists ADP 2.5µmol/l, Collagen 4µg/ml and Adrenaline 5µmol/l. The results are expressed as means ± S.D.

dependent inhibition of platelet aggregation. Responses to adrenaline were mean 40±6% at 10 mins, ADP mean 30±6% at 10 mins and collagen mean 28±6% at 10 mins. However, the important observation seen in these in vitro experiments was that the same pattern of response in order of magnitude was noted irrespective of final drug concentrations (adrenaline>> ADP> collagen). These observed results correlated very well with the experimental findings for D-pen in vitro.

5.2.3 The effects of pHMPSA and L-cysteine singly or in combination, on platelet aggregation inhibition of normal platelets stimulated with ADP, adrenaline and collagen

In the previous in vitro experiments inhibition of platelet aggregation related to D-pen treatment indicates a possible thiol blockade of SH groups on RA platelets. Since blood platelets contain SH groups on their surface receptors experiments were broadend using fresh healthy normal platelets incubated with pHMPSA a non penetrating SH blocking agent. This was to determine whether any platelet aggregation inhibition developed after the addition of pHMPSA when stimulated by the agonists ADP, adrenaline and collagen, and to assess whether this inhibition was due to a blockade of the platelet surface SH groups as related to the D-pen effect.

Initial experiments were performed using various times of incubation and concentrations of pHMPSA. However higher concentrations appeared to damage the platelet membrane and sensitivity of the platelets in the test system was impaired resulting in no data being generated. The established final concentration used in the experiments with pHMPSA and L-cysteine was (10^{-4}M) and incubation time was exactly 1 minute prior to stimulation with either ADP, adrenaline or collagen. Standardisation of aggregation was accomplished by examining the aggregation pattern at 5, 10, 15, 20, 25 and 30 mins respectively.

Figs.5.4.a, 5.4.b and 5.4.c show that the blocking of surface SH groups of fresh normal platelets by incubation with the SH blocking agent pHMPSA (10^{-4}M) resulted in maximum inhibition (>90%) of platelet aggregation for ADP, adrenaline and collagen, and that the addition of L-cysteine did not restore the platelets to a normal aggregation response. Platelet aggregation was observed over a 30 minute period at 5 minute intervals, but as shown in Fig.5.4.a inhibition was almost maximal in 5 minutes. Untreated controls showed some inhibition (up to approximately 30%) due to the loss of sensitivity.

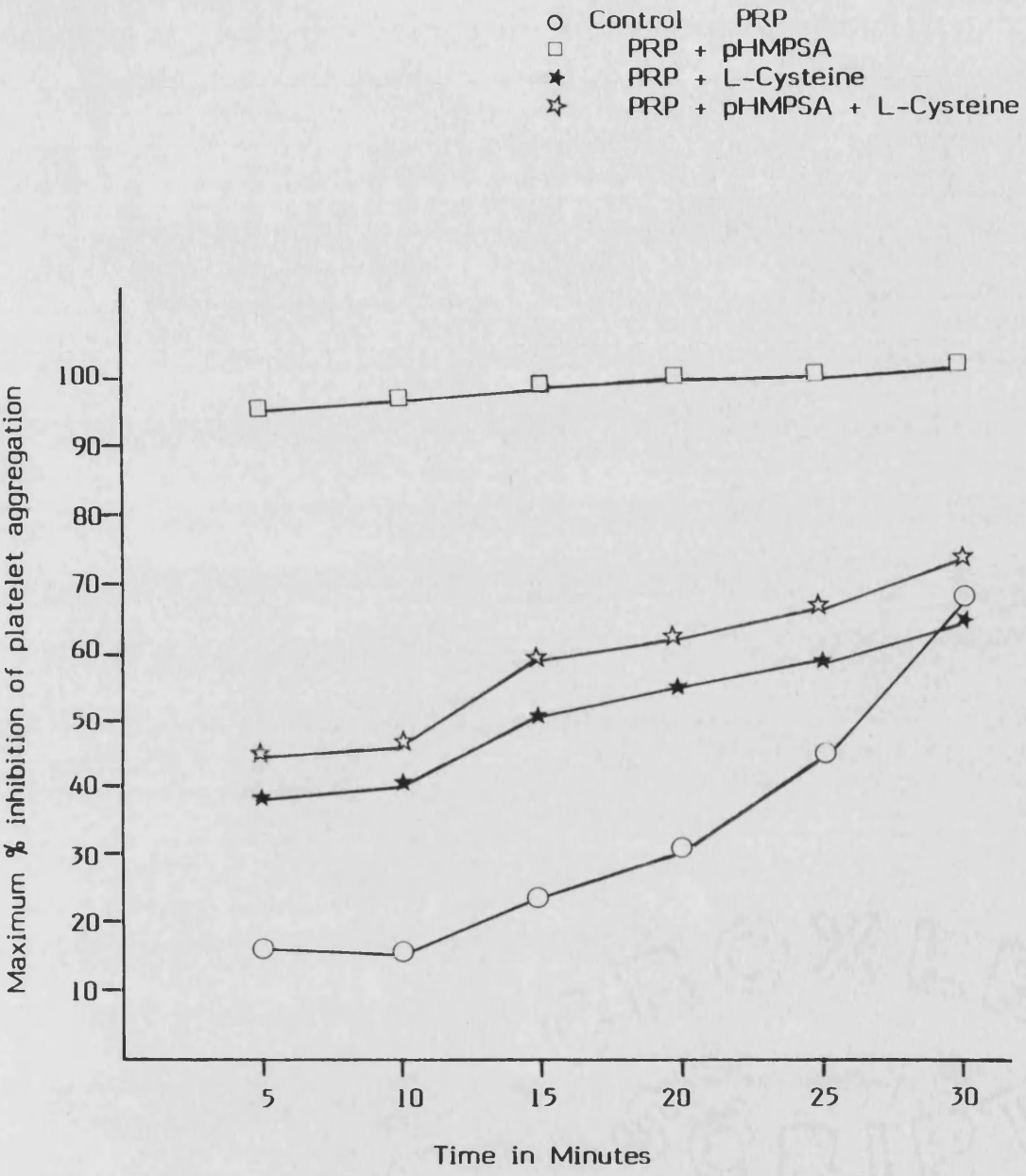


Fig.5.4.a The effects of p-hydroxymercuriphenylsulphonate (pHMPSA)100 µM, and L-Cysteine 100µM, singly or in combination on the % inhibition of Platelet aggregation of normal citrated platelet rich plasma (PRP) produced by A.D.P. 2.5µmol/l, compared to non treated (PRP). The results are expressed as mean ± S.D. (n = 5). The S.D. bars are omitted for clarity.

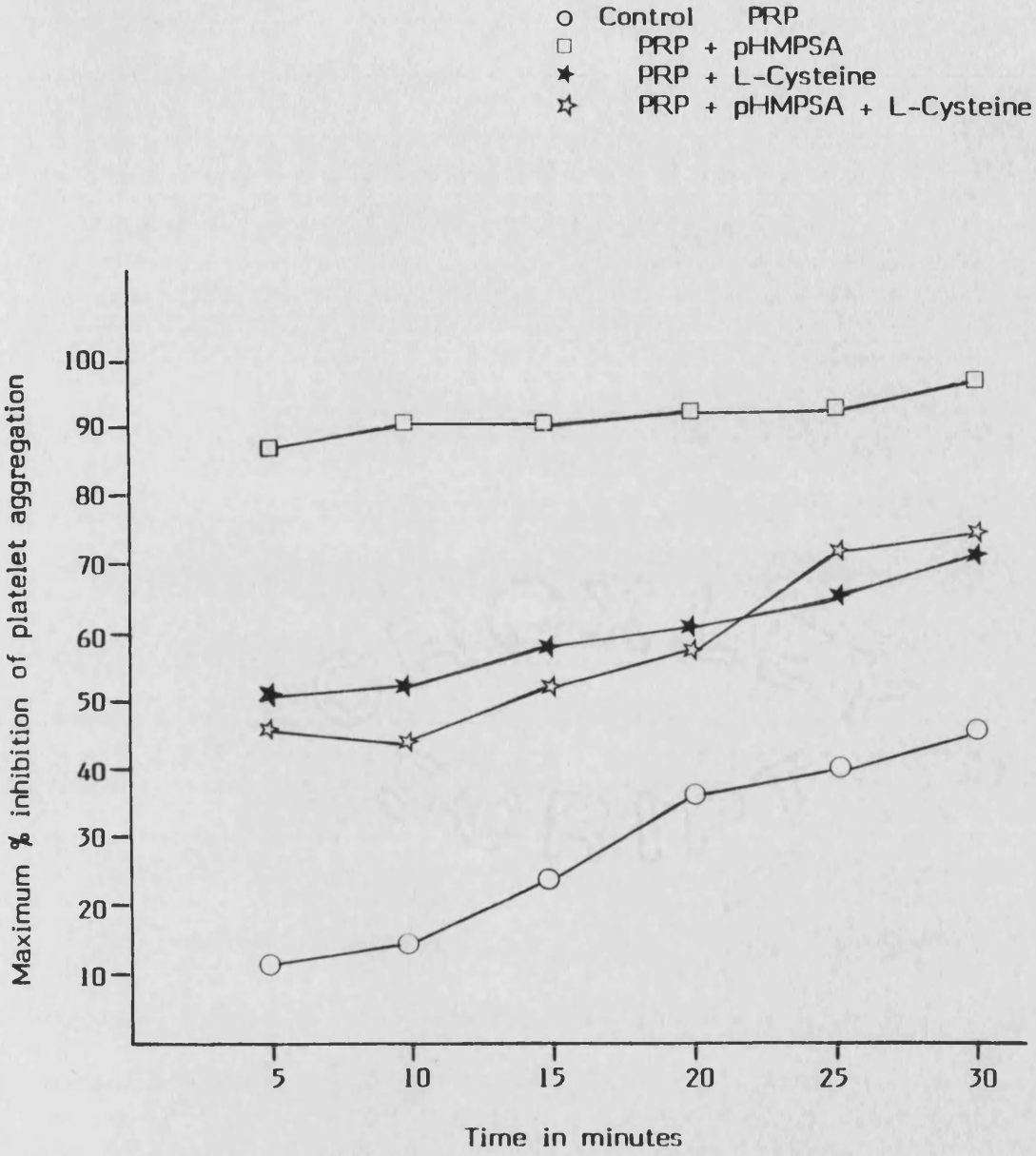


Fig .5.4.b The effects of p-hydroxymercuriphenylsulphonate (pHMPSA) 100 µM, and L-Cysteine 100 µM, singly or in combination on the % inhibition of Platelet aggregation of normal citrated platelet rich plasma (PRP) produced by Adrenaline 5µmol/l, compared to non treated (PRP). The results are expressed as mean ± S.D. (n =5). The S.D. bars are omitted for clarity.

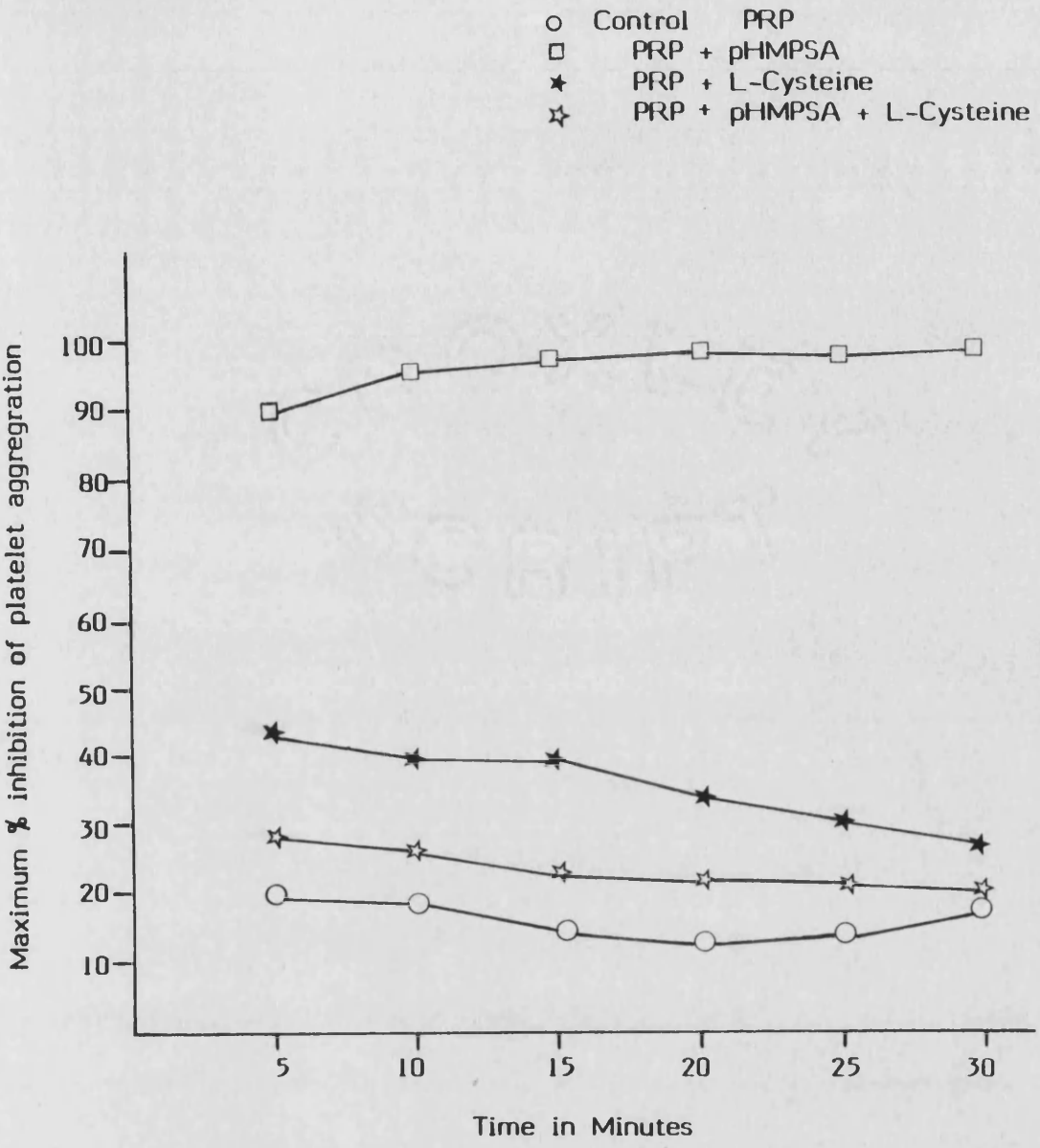


Fig.5.4.c The effects of p-hydroxymercuriphenylsulphonate (pHMPSA) 100μM, and L-Cysteine 100μM, singly or in combination on the % inhibition of Platelet aggregation of normal citrated platelet rich plasma (PRP) produced by Collagen 4μg/ml, compared to non treated (PRP). The results are expressed as mean± S.D. (n = 5). The S.D. bars are omitted for clarity.

5.2.4 The effects of dithiothreitol on fresh RA-D-pen treated platelets and the consequent responses of platelet aggregation to adrenaline

The aim of this investigation was to determine if the altered platelet aggregation response found with RA-D-pen treated platelets could be restored by the addition of dithiothreitol, a powerful reducing agent able to reverse any blocking of SH-groups on the rheumatoid platelets. Experimental evidence suggests that the thiol-disulphide status of platelets is causally related to aggregation and that agents react with thiol groups such as pHMPSA inhibit aggregation.

Various initial experiments were performed to determine incubation times, concentration of dithiothreitol and adrenaline to be used in normal and patient groups. From these observations the incubation times performed were 1 minute, 2 minutes, and 5 minutes. Final concentrations of dithiothreitol were 0.5 mM and 1.0 mM and the concentration of adrenaline for stimulating platelet aggregation was 5µmol/l.

The results in Table 11 show that in vitro pre-incubation of RA-D-pen platelets with DTT brought about a significant restoration of the response to adrenaline. Aggregation responses increased from a mean 12±3% platelet aggregation to a mean level of 56±11% in RA-D-pen platelets + DTT 0.5 mM and

mean of 61±12% with 1.0 mM DTT. Although their aggregability was not restored to the mean values of normal controls 88±8%, the increase was still highly significant ($P < 0.01$). These results are suggestive of a possible thiol blockade by D-pen affecting platelet surfaces. Addition of higher concentrations of DTT caused a progressive but slow platelet aggregation within the test system, starting after a lag phase of four to five minutes.

Patient Groups	R.A. D-pen treated No DTT (n=5)	R.A. D-pen treated +0.5mM DTT (n=5)	R.A. D-pen treated +1.0mM DTT (n=5)	Normal controls +0.5mM DTT (n=5)	Normal controls No DTT (n=5)
1 Minute	12 ± 3	56 ± 11*	61 ± 12	83 ± 8	88 ± 8**
2 Minutes	16 ± 4	49 ± 7	51 ± 8	80 ± 8	85 ± 10
5 Minutes	9 ± 4	42 ± 9	45 ± 9	71 ± 9	76 ± 9
Incubation time Dithiothreitol (DTT)	% Platelet aggregation responses to agonist 5μ mol/l Adrenaline				

Table 11 The effect of Dithiothreitol (DTT) 0.5mM, 1.0mM on Platelet aggregation responses to 5μ mol/l of Adrenaline in R.A. D-pen treated patients compared to normal controls. The results are expressed as mean ± S.D. (n=5). Statistical analysis shows:- a significant difference between R.A. D-pen (no DTT) vs R.A. D-pen (+ DTT)* ($P < 0.01$) and R.A. D-pen (no DTT) vs Control (no DTT)** ($P < 0.001$).

CHAPTER SIX:

DISCUSSION

The results presented in this thesis reveal a significant number of abnormal platelet parameters in patients with rheumatoid arthritis, including an increase in total number and a higher degree of platelet activity (increased B-TG and hypersensitivity to platelet agonists in vitro). These observations together with a higher platelet turnover are consistent with an increased thrombocytosis in rheumatoid arthritis. These findings are discussed in the first part of this chapter.

Following treatment with D-penicillamine (D-pen) the observations of increased thrombocytosis returned to a more normal level, but with clear cut differences in the platelet response. There was total abolition of the aggregatory response to adrenaline, with reduced sensitivity to both ADP and collagen, platelet alpha$_2$ adrenoceptors were also reduced. These findings will be examined and discussed in the second part of this chapter.

Role of platelets in rheumatoid arthritis

Thrombocytosis is a common finding in patients with active and early rheumatoid arthritis (Bean, 1965; Pazdur & Kopec, 1970; Selroos, 1972; Hutchinson, Davis & Jayson, 1975; Hernandez, Rowan, Kennedy & Buchanan, 1975; Valenti, Chianese, Tirri & Giordono, 1979; Colli, Maderna, Tremoli, Colombo & Canesi, 1982). Its presence has been observed in a significant proportion of patients and appears to correlate

with disease activity (Hutchinson, Davis & Jayson, 1975). However, the mechanism underlying the peripheral thrombocytosis occurring in rheumatoid arthritis has not been elucidated. This present study confirms the findings of thrombocytosis in rheumatoid arthritis, but of greater importance lends supportive evidence that the rheumatoid platelets have both abnormal platelet parameters and functions.

In principle an elevated platelet count in disease may be attributable to either increased life span, increased production or changed distribution of platelets within the circulation. As will be discussed, the platelet changes observed in rheumatoid arthritis in this study would support the theory that the elevated platelet count is due to increased production involving excess utilisation and consumption during the inflammatory process.

At one stage thrombocytosis in rheumatoid arthritis had been attributed as secondary to blood loss and iron deficiency anaemia. Pazdur & Kopec, (1970) and Hutchinson, Davis & Jayson, (1975) proved that there was no correlation between the increase of platelet count and degree of anaemia. Indeed, although observations in this study showed a mean lower haemoglobin level in all three disease states, there was no apparent correlation between the anaemia and thrombocytosis. Normo-or hypercellularity of bone marrow is known to occur in the majority of rheumatoid patients (Selroos, 1972); and the

life span of circulating platelets is determined by physiological senescence and by random destruction of thrombocytes taking part in continuous repair of microdamage to the vessel walls, or in inflammatory repair.

The investigation presented in this thesis on platelets and platelet parameters demonstrates that the major population of platelets in rheumatoid arthritis is young and small (microthrombocytes) which are functionally more active than ordinary or older platelets. These platelet populations showed in aggregation experiments an increased sensitivity to low concentrations of ADP and especially collagen. These findings are consistent with the hypothesis that platelet changes are due to increased platelet turnover leading to young and hyperactive platelets, with old "spent" platelets being also present in the circulation. Platelet numbers and parameters were measured and a significant difference noted with platelet count, mean platelet volume and platelet crit in the rheumatoid groups compared to normal. These additional observations also support the theory that the major platelet populations in active rheumatoid arthritis are young, small and hypersensitive.

When platelet sizing techniques became available it was appreciated that there were considerable differences in platelet sizes. The mean platelet volume is known to increase somewhat with time post-venesection (Giles, 1981); and the platelet crit increases similarly. However, as conditions

for collecting blood and performing the analysis were the same for all groups in this study, similar time-dependent changes in the mean platelet volume and platelet crit would be expected to have occurred in all the samples.

Histograms showing platelet volume distribution were plotted and by measuring their relative surface area the percentage of microthrombocytes and megathrombocytes present were calculated. The rheumatoid groups all showed an increased percentage of microplatelets and a lower number of megathrombocytes resulting in a mean platelet volume lower than that found in normal subjects. In the normal population a non-linear inverse relation between platelet count and mean platelet volume has been found (Levin & Bessman 1983), the low mean platelet volume found in rheumatoid thrombocytosis may be an extrapolation of this relation. The introduction of density gradient techniques by Karpatkin (1969) demonstrated differing populations of platelets which he believed to result primarily from an ageing process, the lighter platelets being the younger and the cells becoming more dense as they grew older. He supported this theory by pulse labelling experiments using (^{75}Se) Selenomethionine. However, more recent experiments using cohort labelling suggest that this is not the case (Paulus, Breton-Gorius, Kinet-Denoel & Boniver, 1974; Penington, Streatfield & Roxburgh, 1976; Corash & Shafer, 1982). These studies suggest that platelets of varying density are released simultaneously from the bone marrow, thus making the

possibility that differences between the three types of megakaryocytes represent a major source of heterogeneity of platelets. The data presented with mean platelet volume analysis would support this postulate in that different megakaryocyte ploidy classes release platelets of varying densities that are not altered in the circulation. Thus, the less dense platelets found in rheumatoid arthritis derived from megakaryocytes with higher ploidy, 32-64n, (Firkin, 1985) and accompanied with thrombocytosis may be extremely important. They, therefore, may function in adhesion and aggregation, or in providing fibrillar bayonet type platelets that are important for their interaction with other cells in promoting inflammation and the formation of fibrin (Firkin, 1985).

Blood platelets may participate in the initiation and progression of rheumatoid arthritis in two ways. First, where platelets accumulate locally at points of endothelial injury, microthrombi (containing platelet aggregates and fibrin) are formed and may therefore be predisposed to enhanced platelet destruction, hence a shortened survival (Vollersten, Fuster, Conn, Luthra, McDuffie, Bowie & Ilstrup, 1982). Moreover, the increased turnover of fibrinogen in rheumatoid arthritis (Conn, McDuffie, Kazmier, Schroeter & Sun, 1976) and deposits of fibrin may be considered indicative of occult activation of intravascular coagulation in this disease, thus arguing against prolongation of platelet survival (Bennett, Eddie-Quartey &

Holt, 1972; Selroos & Wegelius, 1973; Hutchinson, Davis & Jayson, 1975).

The hypothesis that blood platelets continuously release the contents of their storage granules while in the circulation has been proposed (O'Brien, 1978). This hypothesis is supported by various conditions in which there is a reduction of the intra-cellular components of platelets. For example in cases of disseminated intravascular coagulation platelets continue to circulate even though there is evidence for a reduction in their spheric granules (Pareti, Capitanio & Mannucci, 1976). A similar mechanism may be operating in rheumatoid arthritis especially as beta-thromboglobulin is only produced in the alpha granules (Ludlam, 1979).

Using a radioimmunoassay, measurements of beta thromboglobulin in the rheumatoid patients showed a significant increase in plasma levels compared with healthy controls. According to Ludlam, Moore, Bolton, Pepper & Cash (1975); Ludlam, (1979); Zahavi & Kakkar, (1980) the plasma concentration of B-TG is assumed to reflect platelet release reaction, as well as platelet turnover (Doyle, Chesterman, Cade, McGready, Rennie & Morgan, 1980). It is a platelet-specific marker and shows direct evidence of enhanced in vivo platelet activity and release reaction. This increase in platelet production may have some relevance to rheumatoid arthritis in that platelet activation either induced by circulating immune complexes or increased sensitivity to

collagen, may promote both platelet aggregation and release from these platelets of cationic inflammatory mediators such as cathepsin, thromboxane A_2 and connective tissue activator peptide (Castor, Ritchie, Scott & Whitney, 1977; Smith & Castor, 1978), which may contribute to the rheumatoid vascular inflammatory state. This suggestion can be explained by a binding of IgG-containing immune complexes via the platelet Fc receptor associated with phagocytosis and will in turn lead to platelet release and the formation of circulating platelet aggregates, which are subsequently sequestered in the spleen. This possibility suggested, is supported by observations discussed by Pfueller & Luscher, (1972).

Secondly, exposure of the subendothelium stimulates the platelets to release subcellular constituents including ADP and beta-thromboglobulin (Holmsen, 1975); and to synthesise thromboxane A_2 from arachidonic acid (Marcus, 1978). It is interesting to note that the increased sensitivity of rheumatoid platelets to ADP and particularly collagen observed in this project may have important consequences leading to tissue damage, especially of endothelial cells, or impaired fibrinolytic mechanisms in rheumatoid arthritis.

Structural determinants both in the collagen molecule and on the platelet membrane that mediate both adhesion and aggregation may well help to accelerate this process. An understanding of the interaction of the physiological

agonists, for example, collagen, ADP and adrenaline with platelet membranes and the mechanism of platelet activation induced by these agonists, may prove to be very important in the pathogenesis of disease states. Most of these agonists have been reported to rely upon fibrinogen (elevated in most rheumatoid patients) binding to platelet receptors as an essential requirement for platelet agregation (Bennett & Vilaire, 1979; Peerschke, 1982) and collagen appears to play a vital role in the interaction between subendothelium and platelets. This interaction is thought to be crucial to the physiology of the primary haemostatic plug as well as to the role of platelets in disease states.

Collagen induced platelet aggregation is a complex process thought to involve several different mechanisms (Nyman, 1977). This author has shown that collagen from various sources as well as different concentrations are capable of activating aspirin inhibited platelets, thus suggesting that there are at least two mechanisms of collagen induced platelet activation, one dependent, and the other independent of arachidonic acid metabolism. This is supported by Best, Holland, Jones & Russell, (1980) who postulated that collagen can apparently induce aggregation and secretion via two pathways, one involving thromboxane production, the other by a direct mechanism independent of thromboxane biosynthesis.

In this context, drugs that inhibit platelet cyclooxygenase activity such as aspirin and other NSAID's have attracted a great deal of attention since they were first shown to inhibit release reaction and some platelet aggregation. However, more recent studies (Kinlough-Rathbone, Cazenave, Packham & Mustard, 1980) have shown that although aspirin inhibits aggregation it does not affect platelet adhesion to collagen or the release reaction of adherent platelets. At high doses aspirin in fact blocks endothelial prostacyclin synthesis and can therefore potentiate platelet aggregation in vivo. Since the collagen-induced release reaction is also unaffected by systems that remove ADP, it has been suggested that platelets adhering to collagen may be capable of undergoing the release reaction by a third pathway which is independent of prostaglandins and thromboxane formation and release of ADP (Kinlough-Rathbone, Cazenave, Packham & Mustard, 1980). The increased sensitivity found in rheumatoid platelets to concentrations of collagen may therefore imply enhanced activity of this pathway in sustaining inflammation.

In addition to these compounds, activated platelets release another important mitogenic factor, platelet derived growth factor (PDGF). This can induce the migration of smooth muscle cells, and stimulation of the normal endothelium adjacent to the damaged area. This can result in the synthesis of prostacyclin, a potent inhibitor of platelet aggregation, hence the balanced equilibrium that normally exists between

vessel wall endothelium and platelets break down, more platelets are stimulated resulting in increased inflammatory mediators being released and hence thrombocytosis.

Although the lipid composition and architecture of the platelet plasma membrane undoubtedly are critical in pathophysiological processes, precise relationships between platelet membrane, lipid structure and platelet function remain unknown. Interaction of the platelet plasma membrane with either particulate stimuli (subendothelial collagen) or soluble stimuli (ADP, adrenaline) initiate profound changes in membrane lipids including (a) the development of procoagulant activity, so called platelet factor 3, which is thought to be associated with absorption of activated factor V on the platelet surface (Marcus, 1978); (b) an increase in the turnover of phospholipids, including phosphatidyl inositol (Marcus, 1978); and (c) the hydrolysis and mobilisation of arachidonic acid from phospholipids with consequent synthesis of biological active eicosanoids (Marcus, 1978).

Thus the platelet is a cell evolved to maintain the integrity of the vascular compartment and as would be expected has an intimate association with humoral and cellular elements in the blood concerned with repair, defence and nutrition, although the functional attributes and properties may be more important for some aspects compared to others. Nevertheless, the platelet should not be thought of as a cell in isolation,

it has a particular affinity and integration with cellular elements of the vessel wall and humoral elements concerned with maintenance, surveillance and repair of vascular integrity and more peripherally with the inflammatory response and the body's defence system. In this respect, alterations to platelets in numbers, parameters, increased turnover and sensitivity may have important implications in the rheumatoid disease process.

Indeed, the therapeutic effect of D-pen in diseases such as rheumatoid arthritis could be to inhibit those stimuli by altering platelet function at the membrane and receptor level in conjunction with impairment of neutrophil polymorphs, monocytes, macrophages and lymphocytes.

Effects of D-penicillamine on platelets in rheumatoid arthritis.

D-pen is a valuable drug used for the treatment of the autoimmune disease-rheumatoid arthritis (Multicentre trial group, 1973). The explanation for its capacity to suppress rheumatoid inflammatory disease, however, remains generally unclear. D-pen has been shown to have wide ranging activity in a variety of in vitro biological systems. The relevance of these activities to its clear clinical anti-rheumatic activity has been discussed at length but with few positive conclusions.

Following D-pen therapy the results presented in this thesis reveal a trend to more normal platelet function, presumably secondary to improvement in disease activity. Initial observations showed rheumatoid platelets after treatment with D-pen to have impaired aggregation responses to ADP, and collagen with almost total inhibition of the adrenaline response. This dramatic effect prompted the investigation of alpha$_2$ adrenoceptor function on blood platelets and the interaction of D-pen with the platelet surface in vitro.

According to the generally accepted concept, platelets normally circulate in a "resting" state and are recruited for functional performance by contact with a foreign surface, or by being acted upon by specific soluble agents functioning as activators. The activation which follows is mediated by specific reactions at the membrane level leading to the products of intracellular "second messengers" that transfer initial contact into a functional response. It follows from this that the properties of the plasma membrane, its receptors and of the intracellular organelle membranes are highly relevant to these functions.

D-pen can modulate mononuclear cell functions via effects on cell membranes. Since these are poorly understood this present study has investigated the effects of D-pen therapy on platelets, cells with well defined receptors coupled to a clear cut response-aggregation. Human platelets can take up and respond to adrenaline and other catecholamines.

Adrenaline activates platelets by binding to specific alpha $_2$ adrenergic receptors, by inhibiting adenylate cyclase and by promoting Ca^{2+} translocation. An understanding of the mechanism of platelet-adrenaline interaction is important in order to study the nature of peripheral alpha$_2$ adrenoceptors and their pharmacological manipulation, also the coupling of the adrenergic stimulus to its receptor and the intracellular responses leading to platelet activation. As will be discussed, following D-pen therapy platelet aggregation in response to adrenaline showed almost total inhibition, perhaps this may be due to an effect on alpha$_2$ adrenoceptors.

In an attempt to relate drug effects more directly to alpha$_2$ receptors radio-ligand binding studies using (^{3}H)yohimbine were performed. Most of the binding studies on alpha$_2$ adrenergic receptors have been performed with lysed platelet membrane preparations which precludes the study of the coupling between ligand binding and the function of intact platelets. However, in this study intact, discoid functional platelets were used as proposed by the method of Boon, Elliot, Grahame-Smith, Outlaw & Stump, (1981) thus removing any anomaly of lysed ruptured platelet membranes interfering with receptor mechanisms. Radioligand binding is a technique that allows one to quantify adrenergic receptors (independent of any response mediated by those receptors) and allows a direct test of the hypothesis that D-pen is possibly associated with altered adrenergic receptors. Alpha$_2$ adrenoceptor binding capacity was moderately

decreased in the RA-D-pen treated platelets compared to both healthy and RA-NSAID platelets. This reduction in receptor number could contribute to the reduced response to adrenaline mediated platelet aggregation found in RA-D-pen treated platelets. The intact aggregatory response to ADP and the absence of a gross reduction in the aggregation response to collagen are against a generalised effect of circulating anti-aggregatory prostaglandins or other factors. However, there is a marked dissociation between the modest reduction of $alpha_2$ receptors binding sites and the virtually total inhibition of the aggregatory response to adrenaline in a functional study. This may be due to several different factors:- including, (a) altered adrenaline concentrations, (b) (^3H)yohimbine binding may not accurately reflect adrenaline binding or (c) $alpha_2$ adrenoceptors are ineffectively coupled to post receptor mechanisms.

Concentrations of adrenaline required to produce aggregation in platelet rich plasma are several orders of magnitude higher than those found in the circulation in vivo. Partly for this reason, the physiological relevance of this receptor mediated response is undetermined as the factors responsible for inter and intra individual variation in platelet responsiveness to adrenaline have not been fully assessed. Studies which have reported altered adrenaline aggregation of platelets with sex, age and ischaemic heart disease have used different methods of assessing the aggregation, and often using only a single concentration of agonist without

full evaluation of overall dose response relationships (O'Brien, 1964b; Yokoyama, Kawishima, Sakamoto, Akita, Okada, Mizutani & Fukuzaki, 1983).

Alternatively, the receptors labelled by (^{3}H) yohimbine may represent only a proportion of the population of functional alpha$_2$ adrenoceptors. Thus, the concentration of binding sites identified by (^{3}H) dihydroergocryptine which pharmacologically appear to have characteristics of alpha$_2$ adrenoceptors is two fold greater than those labelled with (^{3}H) yohimbine in the same platelet membrane (Motulsky & Insel, 1982). Another possible explanation is that (^{3}H) dihydroergocryptine is not selective for alpha$_2$ adrenoceptor subtype and may also label other receptor populations. Morever, it is associated with a high level of non-specific binding to membrane components compared to my non-specific binding studies with (^{3}H) yohimbine. However, it is not possible to make any definite statements regarding such differences between adrenaline functional aggregation and alpha $_2$ receptors until further information is available regarding the labelling of alpha$_2$ receptors with (^{3}H) yohimbine and (^{3}H) dihydroergocryptine.

Observations that inhibition of the alpha receptor with (^{3}H) yohimbine blocked the adrenaline effect is in keeping with the reports of other investigators who showed that the action of adrenaline on platelets is mediated through an alpha$_2$ receptor (Scrutton & Wallis, 1981). However, Yu & Latour,

(1977), and Kerry & Scrutton,(1983) conclude that the effect of adrenaline is possibly dependent upon a balance between alpha receptor agonists inhibiting adenylate cyclase and beta receptor agonists stimulating it. Alternatively a recent report by Figures, Strimpler, Scearce, Mills, Wachtfogel, Colman & Colman, (1984) show that adrenaline increases the binding of ADP to platelets and indicates that this has to be considered as another pathway by which adrenaline may exert its potentiating effect on ADP, the main pivot for platelet aggregation.

The dissociation between $alpha_2$ adrenoceptor status assessed by (^3H)yohimbine binding and functional aggregation may have yet another explanation. Only part of the adrenaline induced aggregation response may be directly dependent on $alpha_2$ adrenoceptor stimulation. A recent study by Connolly & Limbird, (1983) indicates that whilst increased Na^+ concentration enhances platelet aggregation induced by adrenaline, there is no effect of Na^+ on adrenaline induced inhibition of PGE stimulated cyclic AMP production in intact platelets. This differential modulatory influence suggest that at least part of the stimulus response coupling mechanism for the functional response to adrenaline is separate from that for adrenaline induced, $alpha_2$ mediated inhibition of cyclic AMP production which is the only direct primary biochemical consequence of $alpha_2$ adrenoceptor activation so far identified in the platelet.

An understanding of the interaction of physiological agonists e.g. ADP, adrenaline and collagen with platelet membrane receptors and the mechanism of platelet activation induced by these agonists may prove to be important in the pathogenesis of many disease processes.

The catalytic unit of the adenylate-cyclase system is known to be regulated by membrane-bound receptors via guanine nucleotide regulatory (G) proteins (Rodbell, 1980). In platelets, agonists such as prostacyclin bind to the receptors and stimulate the catalytic unit of the enzyme via a stimulating (G) protein. However, other agonists for example, adrenaline, inhibit the catalytic unit, and do so via specific receptors which activate an inhibitory G protein (Gi); (Hildebrandt, Sekura, Codina, Iyenger, Manclark & Birnbaumer, 1983). Both effects are enhanced by GTP and inhibited by N-ethylmaleimide an SH-blocking agent (Jakobs, Lasch, Minuth, Aktories and Schultz, 1982). Treatment with N-ethylmaleimide prevents inhibition of adenylate cyclase and stimulation of GTPase by adrenaline in platelets without, however, significantly altering either uninhibited levels of adenylate cyclase activity or the extent of affinity for (3H)yohimbine. Thus under these conditions N-ethylmaleimide uncouples the $alpha_2$ adrenoceptors from adenylate cyclase (Jakobs, Lasch, Minuth, Aktories & Schultz, 1982). One can postulate that D-pen may be uncoupling the receptor mechanisms in a similar way and thus blocking the action of adrenaline.

Similarly reductions of sensitivity to ADP were noted 2-3 months post D-pen therapy and one may postulate that this effect arises by a similar mechanism on the Gi protein. If, true, there must be a different coupling chemistry for alpha$_2$ adrenoceptor and ADP receptor to Gi protein, or adrenaline response is synergistically involved with both alpha$_2$ adrenoceptors and Gi protein effects. This hypothesis would help to explain the difference found between ADP and adrenaline responses in the aggregation inhibition experiments.

The in vitro functional response may not accurately reflect platelet alpha$_2$ adrenoceptor function in vivo where the role of adrenaline may be to promote aggregation induced by a number of other influences acting in concert. It is possible that measurement of total concentration of alpha$_2$ adrenoceptors may be too crude an estimate of adrenoceptor status. Thus, it has been demonstrated that the alpha$_2$ adrenoceptor, in common with other adenylate-cyclase linked receptors, may exist in two interconvertible agonist affinity states (U'Prichard, Mitrius, Kahn & Perry, 1982). It is possible that the guanine nucleotide sensitive high affinity state may relate more directly to the functional activity of the receptor. Hence subtle changes in these affinity states of the adrenoceptor may be more relevant in relationship to adrenaline induced aggregation.

Other workers indicate that aggregation and dense granule secretion are separate steps in the sequence of responses, only distinguished by different thresholds of concentration of the intracellular messengers. Such a proposal has previously been described "the basic platelet reaction " (Holmsen, 1974; Holmsen, 1978; Holmsen, 1980) in which one and the same second messenger triggers responses. At present several intracellular messengers are believed to function such as Ca^{2+}, diacylglycerol and inositol-1-4-5-triphosphate (Berridge, 1984).

It is possible that adrenaline may affect the level of one or more intracellular messengers, for example, it is known that adrenaline lowers cAMP, perhaps diacylglycerol inositol-1-4-5-triphosphate and/or Ca^{2+} are also decreased or increased respectively, thereby controlling the different threshold for aggregation and secretion. Perhaps this process is having a dramatic effect on platelet aggregation associated with inflammatory events found in rheumatoid arthritis.
Although the synergistic stimulation by adrenaline of thrombin induced platelet aggregation has been well documented (O'Brien, 1964b), the mechanism remains unknown. In platelet rich plasma, adrenaline induces a primary and secondary aggregation and secretion without causing shape change. Of these adrenaline induced responses only the primary aggregation is independent of the cyclooxygenase pathway (Scrutton & Wallis, 1981). Adrenaline could be regarded as proaggregatory (positive agonist interaction),

by enhancing the synthesis of thromboxane A_2, or alternatively, by decreasing the level of intracellular cAMP (Jakobs, Saur & Schultz, 1976; Aktories & Jakobs, 1981; Cameron & Ardlie, 1982). The abolition of adrenaline mediated function by D-pen could suppress any such reaction that required the synergistic action of this hormone. These findings may be relevant to the enhanced aggregation found in rheumatoid arthritis which diminished following treatment with D-pen, and loss of aggregatory response adrenaline >> ADP >> collagen.

Some recent studies have shown that the level of intracellular free Ca^{2+} in platelets may be regulated through the level of cAMP (Zavoico & Feinstein, 1984). Another possibility is that diacylglycerol and Ca^{2+} function as synergistical second messengers, and that adrenaline changes the ratio between them. In order to elucidate the mechanisms of adrenaline action further studies are required in which synergistically induced aggregation and secretion, and alteration in intracellular free Ca^{2+}, cAMP and phospholipid metabolism are measured simultaneously.

Despite extensive studies on the mechanism of action of adrenaline, the cellular events leading to its end function, for example, activation of contractile proteins are largely unknown. Presumably, after receptor stimulation platelet aggregation can be amplified by other mechanisms such as ADP release or thromboxane A_2 synthesis, so that platelet

aggregation does not directly reflect receptor involvement, such a concept could explain the discrepancy found between the loss of adrenaline - induced functional aggregation compared to the modest reduction in platelet alpha$_2$ receptors in RA-D-pen platelets.

In this study assessment was made of the ability of adrenaline, ADP and collagen to induce malonyldialdehyde (MDA) release from the platelets of normal, RA-NSAID and RA-D-pen treated subjects. MDA is a major breakdown product of the metabolism of arachidonic acid to prostaglandin and thromboxane. Compared with normal controls MDA production was reduced both in the RA-NSAID and RA-D-pen treated platelets. This was not surprising as all patients had at some stage received various NSAID's which are known inhibitors of cyclooxygenase. It is interesting to note that the MDA levels of the NSAID's group and the pre-D-pen treated group (also NSAID's) showed a significant difference. A possible explanation for this is that five more patients in the pre-D-pen group were receiving indomethacin, a potent inhibitor of the cyclooxygenase pathway compared to the NSAID's group who included only two patients who had been prescribed this drug, no patient was receiving aspirin. The difference between the other NSAID's and aspirin is that aspirin irreversibly acetylates the platelet enzyme cyclooxygenase resulting in no MDA formation, since platelets cannot resynthesize cyclooxygenase the defect persists for their whole life cycle unlike the effect on other NSAID's where the effect only lasts

several hours. Particularly low levels of MDA were found with adrenaline stimulation of RA-D-pen platelets. The degree of reduction in MDA concentration paralleled the loss of alpha$_2$ adrenoceptor response but was much less than the total inhibition of platelet aggregation in response to adrenaline, these results may be due to involvement of the Gi protein. It is known that adrenaline induces both primary and secondary aggregation of human blood platelets in vitro (Mills & Roberts, 1967). Secondary aggregation is thought to be mediated by ADP released from dense granules and, or, products of arachidonic acid metabolism, whereas primary aggregation seems to result from adrenaline stimulation of platelet alpha$_2$ receptors. It would therefore seem that aggregation although mediated by the arachidonic acid pathway is quite capable of functioning by another pathway.

Current knowledge of platelets participating in the inflammatory process suggests that they participate by their functions of adhesion, release of endogenous platelet specific proteins and aggregation. The inhibitory effect of a drug can cause impairment of any or all of these aspects of platelet function. I elected to study the effect on D-pen on platelet function using an in vitro aggregation technique because it encompassed platelet specific functions of membrane receptor regognition of agonists, release reaction and platelet aggregation. Addition of drug to normal platelets in vitro allowed a better control of experimental variables, such as donor selection, platelet count,

incubation conditions, drug concentrations and solution stability. The in vitro studies agreed with earlier observations made ex vivo of inhibition of aggregation by D-pen, but the overall results were less specific with inhibition of both ADP and collagen as well as the adrenaline response. The latter was however more markedly affected than the other agonists. Differences betwen in vivo and in vitro experiments could relate specifically to the disease state or to the abnormal platelet parameters and turnover found in rheumatoid arthritis. It was also noted that longer incubations did not result in a significant increase or decrease in the inhibitory response. This suggests that D-pen inhibits platelet function by interacting with the platelet membrane where the drug may interfere with the binding of agonists to the specific receptors residing in platelet surface glycoproteins.

Indeed, in vitro experiments with both D-pen and sodium aurothiomalate (both sulphydryl compounds) showed inhibition of platelet aggregation with a similar pattern to that observed with D-pen in vivo. Thus supporting the theory that D-pen is possibly acting as a thiol blocker of SH-groups on the platelet surface. However, blockade of platelet cell surface SH-groups using the irreversible cell surface SH blocker pHMPSA produced a non-specific pattern of inhibition for platelet aggregation with all three agonists despite using a wide range of concentrations. The addition of L-cysteine only had a minimal effect in restoring aggregation,

presumably pHMPSA is too potent a chemical for platelet receptor membranes resulting in non-specificity. However, good evidence for D-pen binding and blocking to platelet receptor mechanisms was achieved with dithiothreitol.

D-pen functioning on receptor mechanisms was determined with in vitro experiments using dithiothreitol (DTT) a powerful reducing agent, which largely restored the response to adrenaline. The most likely explanation for this process is that D-pen binds reversibly with the SH groups on the platelet surface forming disulphides. The addition of DTT appears to reverse this effect, removing the D-pen block and restoring the $alpha_2$ receptor function. The DTT had no apparent detrimental effect on intracellular metabolism.

Concluding remarks

It is tempting to postulate that the loss of adrenaline response on platelets following D-pen treatment may have a modulatory effect on general platelet aggregation acting synergistically, thus modifying inflammation in rheumatoid arthritis. There may also be a general effect of D-pen on Gi proteins and thereby on adenylate cyclase activity. Gi protein is widely distributed and involved in regulating many cells. Potentially therefore, it may have a major impact on many cell types. Thirdly, D-pen may be acting on $alpha_2$ receptors in other sites in the body, but no evidence for this as yet. However, other inflammatory cells express $alpha_2$

receptors including lymphocytes and polymorphonuclear neutrophils (Sanders & Munson, 1985). All these theories remain purely speculative, further studies are obviously very important.

From these observations there is some indication that blood platelets might be involved in the pathogenesis and progression of rheumatoid arthritis. In this respect use of drugs like D-pen that, among other actions, inhibit platelet aggregation seem appropriate.

A combination of physiological stimuli may well be the modulatory mechanism in vivo. Exploration of synergistic actions on inhibition of platelet activation should be undertaken to determine how many of the parallel activation pathways need to be blocked, in order to prevent the complications which promote endothelial damage and inflammation. Such synergy between alpha$_2$ adrenoceptors and other stimuli could be important in regulating other cells relevant to the pathogenesis of rheumatoid arthritis. The unravelling of these pathways has significant implications for therapeutic intervention with pathological states such as rheumatoid arthritis as well as for the understanding of cellular functions beyond platelet physiology.

BIBLIOGRAPHY

Abdou, N.I., Lindsley, H.B., Racela, L.S., Pascual, E. and Hassanein, K.M. (1981).
Suppressor T cell dysfunction and anti-suppressor cell antibody in active early R.A.
J. Rheumatol. **8**, 9.

Abraham, E.P., Baker, W., Chain, E., Florey, H.W., Holliday., E.F. and Robinson, R. (1942).
The nitrogenous character of penicillin.
Nature (Lond.) **149**, 356.

Abraham, E.P., Chain, E., Baker, W. and Robinson, R. (1943).
Penicillamine: A characteristic degradation product of penicillin.
Nature (Lond.) **151**, 107.

Aktories, K. and Jakobs, K.H. (1981).
Epinephrine inhibits adenylate cyclase and stimulates GTPase in human platelet membranes via alpha-adrenoceptors.
FEBS. Lett. **130**, 235-238

Albert, D.H. and Snyder, F. (1983).
Biosynthesis of 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (Platelet-activating factor) From 1-alkyl-2-acyl-sn-glycero-3-phosphocholine by rat alveolar macrophages. Phospholipase A_2 and acetyltransferase activities during phagocytosis and Ionophore stimulation.
J. Biol. Chem. **258**, 97-102.

Albert, D.H. and Snyder, F. (1984).
Release of arachidonic acid from 1-alkyl-2-acyl-sn-glycero-3-phosphocholine, a precursor of platelet-activating factor, in rat alveolar macrophages.
Biochim. Biophys. Acta. **696**, 92-101.

Allen, C., Elson, C.J., Scott, D.G.I., Bacon, P.A. and Bucknall, R.C. (1981).
IgG antiglobulins in rheumatoid arthritis and other arthritides relationship with clinical features and parameters.
Ann. Rheum. Dis. **40**, 127-131.

Alspaugh, M.A., Jensen, F.C., Rabin, H. and Tan, E.M. (1978).
Lymphocytes transformed by Epstein-Barr virus. Induction of nuclear antigen reactive with antibody in rheumatoid arthritis.
J. Exp. Med. **147**, 1018-1027.

American Rheumatism Association Co-operating Clinics Committee. (1973).
A controlled trial of gold salt therapy in rheumatoid arthritis.
Arthritis. Rheum. **16**, 353.

Anderson, A.J., Brocklehurst, W.E. and Willis, A.L. (1971).
Evidence for the role of lysosomes in the formation of prostaglandins during carrageenin induced inflammation in the rat.
Pharmac. Res. Commun. **3**, 13-19.

Appleford, D.J.A. and Denman, A.M. (1979a).
Fate of herpes simplex virus in lymphocytes from inflammatory joint effusions. I. Failure of virus to grow in cultured lymphocytes.
Ann. Rheum. Dis. **38**, 443-449.

Appleford, D.J.A. and Denman, A.M. (1979b).
Fate of herpes simplex virus in lymphocytes from inflammatory joint effusions. II. Mechanisms of non-permissiveness.
Ann. Rheum. Dis. **38**, 450-455.

Armitage, P. (1977).
Statistical methods in Medical Research.
Blackwell Scientific Publications.

Bacon, P.A. (1979).
Circulating immune complexes in systemic rheumatoid disease.
Rheumatol. Rehabil. Suppl. **11**, 5.

Bacon, P.A., Cracchiolo, A., Bluestone, R. and Goldberg, L.S. (1973).
Cell mediated immunity to synovial antigens in rheumatoid arthritis.
Lancet **ii**, 699-702.

Balogh, Z., El-Ghobarey, A., Fell, G., Brown, D., Dunlop, J. and Dick, W. (1980).
Plasma zinc and its relationship to clinical symptoms and drug treatment in rheumatoid arthritis.
Ann. Rheum. Dis. **39**, 329-332.

Barden, J., Mullinay, F. and Waller, M. (1967).
Immunoglobulin levels in rheumatoid arthritis. Comparison with rheumatoid factor titres: clinical stage and disease duration.
Arthritis Rheum. **10**, 228-234.

Barnhart, M.I., Riddle, J.M. and Bluhm, G.B. (1967).
Fibrin promotion and lysis in arthritic joints.
Ann. Rheum. Dis. **26**, 206-218.

Bean, R.H.D. (1965).
Thrombocytosis in auto-immune disease.
Bibl. Haematol. **23**, 43-49.

Becker, A.J., McCulloch, E.A. and Till, J.E. (1963).
Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells.
Nature (Lond.) **197**, 452-455.

Begg, G.S., Pepper, D.S., Chesterman, C.N. and Morgan, F.J. (1978).
Complete covalent structure of human B-thromboglobulin.
Biochemistry **17**, 1739-1744.

Bentfeld-Barker, M.E. and Bainton, D.F. (1982).
Identification of primary lysosomes in human megakaryocytes and platelets.
Blood **59**, 472-481.

Bennett, J.S. & Vilaire, G. (1979).
Exposure of platelet fibrinogen receptors by ADP and epinephrine.
J. Clin. Invest. **64**, 1393-1401.

Bennett, R.M., Eddie-Quartey, A.C. and Holt, P.J.L. (1972).
Fibrin degradation products in rheumatoid arthritis.
Ann. Rheum. Dis. **31**, 388-392.

Berridge, M.J. (1984).
Inositol triphosphate and diacylglycerol as second messengers.
Biochem. J. **220**, 345-360.

Bertino, J.K., Hurd, E.R., Decker, J.T. and Steinberg, A.D. (1973).
A.R.A. symposium: Cytotoxic Drugs.
Arthritis. Rheum. **16**, 79.

Best, L.C., Holland, T.K., Jones,P.B., and Russell, R.G. (1980).
The interrelationship between thromboxane biosynthesis, aggregation and 5-hydroxytryptamine secretion in human platelets in vitro.
Thromb. Haemost. **43**, 38-40.

Billah, M.M. and Lapetina, E.G. (1982a).
Formation of lysophosphatidylinositol in platelets stimulated with thrombin or ionophore A 23187.
J. Biol. Chem. **257**, 5196-5200.

Billah, M.M. and Lapetina, E.G. (1983).
Platelet activating factor stimulates metabolism of phosphoinositides in horse platelets: possible relationship to Ca^{2+} mobilization during stimulation.
Proc. Natl. Acad. Sci. USA. **80**, 965-968.

Billingham, M.E.J. and Davies, G.E. (1979).
Experimental models of arthritis in animals as screening tests for drugs to test arthritis in man.
Anti-inflammatory Drugs.
Handbook of Experimental Pharmacology 50/11.
eds. Vane. J.R. and Ferreira S.H. Springer-Verlag, Berlin.

Bolton, A.E., Ludlam, C.A., Moore, S., Pepper, D.S. and Cash, J.D. (1976).
Three approaches to the radioimmunoassay of human B-thromboglobulin.
Brit. J. Haematol. **33**, 233-238.

Boon, N.A., Elliott, J.M., Grahame-Smith, D.G., Outlaw, T. and Stump. K. (1981).
Binding of (^{3}H)-yohimbine to alpha adrenoceptors on intact human platelets.
Br. J. Pharmacol. **74**, Proc. BPS. 802P.

Born, G.V.R. (1962).
Aggregation of blood platelets by adenosine diphosphate and its reversal.
Nature (Lond.) **194**, 927-929.

Boullin, D.J. & Elliott, J.M. (1979).
Binding of (^{3}H)-dihydroergocryptine to alpha-adrenoceptors on intact human platelets.
Br. J. Pharmacol. **66**, 89-90.

Brass, L.F. and Shattil, S.J. (1982).
Changes in surface bound and exchangeable calcium during platelet activation.
J. Biol. Chem. **257**, 14000-14005.

Brass, L.F. and Shattil, S.J. (1984).
Indentification and function of the high affinity binding sites for Ca^{2+} on the surface of platelets.
J. Clin. Invest. **73**, 626-632.

Brewerton, D.A., Caffrey, M.F.P., Hart, F.D., James, D.C.O., Nicholls, A. and Sturrock, R.D. (1973).
Ankylosing spondylitis and HLA-B27.
Lancet **i**, 904-907.

Britton, M.C. and Schur, P.H. (1971).
The complement system in rheumatoid synovitis. II. Intra-cytoplasmic inclusions of immunoglobulins and complement.
Arthritis. Rheum. 14, 87-95.

Bywaters, E.G.L. (1975).
The historical evolution of the concept of connective tissue diseases.
Scand. J. Rheumatol. 5, 11-29.

Caen, J. (1972).
Glanzmann Thrombasthenia.
Clin. Haematol. 1, 383-392.

Cameron, H.A. and Ardlie, N.G. (1982).
The facilitating effects of adrenaline on platelet aggregation.
Prostaglandins Leukotrienes Med. 9, 117-128.

Camp, A. (1981).
Haematologic toxicity from penicillamine in rheumatoid arthritis.
J. Rheumatol. (Supp.7) 8, 164-165.

Campbell, A.C., Skinner, J.M., McLennan, I.C.M., Hersey, P., Waller, C.A., Wood, J., Jewell, D.P. and Truelove, S.C. (1976).
Immunosuppression in the treatment of inflammatory bowel disease.
Clin. Exp. Immunol. **24**, 249.

Carson, D.A., Lawrance, S., Catalano, M.A., Vaughan, J.H. and Abraham, G. (1977).
Radio immunoassay of IgG and IgM rheumatoid factors reacting with human IgG.
J. Immunol. **119**, 295-300.

Castor, C.W., Ritchie, J.C., Scott, M.E. and Whitney, S.L. (1977).
Connective tissue activation. XI. Stimulation of glycosaminoglycan and DNA formation by a platelet factor.
Arthritis. Rheum. **20**, 859-868.

Catalano, M.A., Carson, D.A., Niederman, J.C., Feorino, P. and Vaughan, J.H. (1980).
Antibody to the rheumatoid arthritis nuclear antigen. Its relationship to in vivo Epstein-Barr virus infection.
J. Clin. Invest. **65**, 1238-1242.

Cavero, J., Gomeni, R., Lefevre-Borg, F. and Roach. A.G. (1980).
Comparison of mianserine with desipramine, maprotiline and phentolamine on cardiac presynaptic and vascular postsynaptic alpha-adrenoceptors and noradrenaline reuptake in pithed normotensive rats.
Br. J. Pharmacol. 68, 321-332.

Chain, E. (1949).
Penicillamine: a characteristic degradation product of penicillin.
"Antibiotics". Florey, Vol 11. Oxford University Press. London.

Chanmougan, D. and Schwartz, R.S. (1966).
Enhancement of antibody synthesis by 6-mercaptopurine.
J. Exp. Med. 124, 363.

Chattopadhyay, C., Chattopadhyay, H., Natvig, J.B., Michaelson, T.E. and Mellbye. O.J. (1979).
Lack of suppressor cell activity in rheumatoid synovial lymphocytes.
Scand. J. Immunol. 10, 309.

Chayen, J. and Bitensky, L. (1971).
Lysosomal enzymes and inflammation with particular reference to rheumatoid diseases.
Ann. Rheum. Dis. **30**, 522-536.

Chesney, C.McI., Harper, E. and Colman, R.W. (1974).
Human platelet collagenase.
J. Clin. Invest. **53**, 1647-1654.

Clare, K.A. and Scrutton, M.C. (1983b).
The properties of $^{45}Ca^{2+}$ uptake into human blood platelets induced by PAF and adrenaline.
Thromb. Haemost. **50**, 41.

Clare, K.A. and Scrutton, M.C. (1984).
The role of Ca^{2+} uptake in the response of human platelets to adrenaline and to 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet-activating factor).
Eur. J. Biochem. **140**, 129-136.

Clark, R. (1983).
Oxidant injury by a penicillamine-copper model system.
Clin. Res. **31**,(2), 448a.

Cohen, P. and Derksen,A. (1969).
Comparison of phospholipid and fatty acid composition of human erythrocytes and platelets.
Brit. J. Haematol. **17**, 359-371.

Colli, S., Maderna, P., Tremoli, E., Colombo, F. and Canesi, B. (1982).
Platelet function in rheumatoid arthritis.
Scand. J. Rheumatol. **11**, 139-143.

Conn, D.L., McDuffie, F.C., Kazmier, F.J., Schroeter, A.L. and Sun, N.C.J. (1976).
Coagulation abnormalaties in rheumatoid arthritis.
Arthritis. Rheum. **19**, 1237-1243.

Connolly, T.M. and Limbird, L.E. (1983).
The influence of Na^+ on the $alpha_2$ adrenergic receptor system of human platelets.
J. Biol. Chem. **258**, 3907-3912.

Corash, L. and Shafer, B. (1982).
Use of asplenic rabbits to demonstrate that platelet age and density are related.
Blood **60**, 166-171.

Crook, D., Collins, A.J., Bacon, P.A. and Chan, I.R. (1976).
Prostaglandin synthetase activity from human rheumatoid synovial microsomes.
Ann. Rheum. Dis. **35**, 327.

Currey, H.L.F. (1978).
Therapeutic alteration of immune responses in rheumatoid arthritis: is it relevant?
J. Roy. Soc. Med. **71**, 275.

Davis, P. (1984).
Penicillamine metal chelates and their possible importance in rheumatoid arthritis - a brief review.
Clin. Invest. Med. **7(1)**, 41-44.

Dawes, J., Smith, R.C. and Pepper, D.S. (1978).
The release, distribution and clearance of human beta-thromboglobulin and platelet factor 4.
Thromb. Res. **12**, 851-861.

Demopoulos, C.A., Pinckard, R.N. and Hanahan, D.J. (1979).
Platelet activating factor. Evidence for 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphoryl-choline as the active component (a new class of lipid chemical mediators.)
J. Biol. Chem. **254**, 9355-9358.

Denman, A.M. (1975).
The viral theory of connective tissue diseases.
Med. Bio. 53, 61-84.

Denman, A.M. (1979).
The macrophage,chronic inflammation and rheumatoid arthiritis. In: "Immunopathogenesis of Rheumatoid Arthritis". eds. Panayi, G.S. and Johnson, P.M. Reedbooks Ltd., Cherstsey. 123-130.

Denman, A.M., Appleford, D.J., Imrie, R.C., Kinsley, M.J., Pelton, B.K. and Schnitzer, T. (1977).
Lymphocytes and virus infections in the connective tissue diseases. In: "Rheumatoid Arthritis". eds. Gordon, J.L. and Hazleman, B.L. North Holland Publishing Co Ltd., Oxford. 233-245.

Denham, M.J,, Fisher, M., James, G and Hassan, M. (1977).
Plasma concentration of B-thromboglobulin in venous and arterial thrombosis.
Lancet i, 1154.

Depper, J.M. and Zvaifler. N.J. (1981).
Epstein Barr virus. Its relationship to the pathogenesis of rheumatoid arthritis.
Arthritis. Rheum. 24, 755-761.

Deutsch, H.F. and Morton, J.I. (1957).
Dissociation of human serum macroglobulins.
Science **125**, 600.

Doyle, D.J., Chesterman, C.N., Cade, J.F., McGready, J.R., Rennie, G.C. and Morgan, F.J. (1980).
Plasma concentrations of platelet-specific proteins correlated with platelet survival.
Blood **55**, 82-84.

Eisen, V. (1969).
Kinin formation and human diseases.
In: "The Scientific Basis of Medicine".
Athlone Press. London. 146-165.

Empire Rheumatism Council Research Sub-Committee. (1960).
Gold therapy in rheumatoid arthritis.
Report of a multicentre controlled trial.
Ann. Rheum. Dis. **19**, 95.

Empire Rheumatism Council. (1961).
Gold therapy in rheumatoid arthritis.
Final report of a multicentre trial.
Ann. Rheum. Dis. **20**,315.

Endresen, G.K.M. (1981).
Investigation of blood platelets in synovial fluid from patients with rheumatoid arthritis.
Scand. J. Rheumatol. **10**, 204-208.

Erslev, A.J. and Gabuzda. T.G. (1975).
Pathophysiology of Blood. **6**, 141.

Evatt, B.L. and Levin, J. (1969).
Measurement of thrombopoiesis in rabbits using ^{75}Selenomethionine.
J. Clin. Invest. **48**, 1615-1626.

Fain, J.N. and Garcia-Sainz, J.A. (1980).
Role of phosphatidylinositol turnover in alpha$_1$ adrenoceptor and adenylate cyclase inhibition in alpha$_2$ adrenoceptor, effects of catecholamines.
Life. Sci. **26**, 1183-1194.

Fauci, A.S., Dale, D.C. and Balow, J.E. (1976).
Glucorticosteriod therapy: mechanisims of action and clinical considerations.
Ann. Intern. Med. **84**, 304.

Fell, H.B. (1978).
Synoviocytes.
J. Clin. Path. **31**, Suppl. (Roy.Coll.Path.) **12**, 14-24.

Ferrell, P.B., Aitcheson, C.T., Pearson, G.R. and Tan, E.M. (1981).
Seroepidemiological study of relationships between Epstein-Barr virus and rheumatoid arthritis.
J. Clin. Invest. 67, 681-687.

Figures, W.R., Strimpler, A.M., Scearce, M., Mills, J.K., Wachtfogel, Y.T., Colman, R.F. and Colman, R.W. (1984).
Role of ADP receptors in epinephrine induced platelet activation.
Fred. Proc. 43, 660(Abst).

Fink, P.C., Piening, U., Fricke, P.M. and Deicher, H. (1979).
Platelet aggregation and aggregation inhibition by different antiglobulins and antiglobulin complexes from sera of patients with rheumatoid arthritis.
Arthritis. Rheum. 22, 896-903.

Firkin, B.G. (1985).
Origin of the platelet. In: "The Platelet and its Disorders".
MTP Press Ltd., 6.

Flower. R.J., (1984).
Macrocortin and the Antiphospholipase Proteins. In: "Advances in Inflammation Research." 8. ed. Weissmann, G. Raven Press, New York. 1-35.

Flower, R.J. (1985).
Background and discovery of Lipocortins.
Agents and Actions. **17**, 255-262.

Forestier, J. (1929).
L'aurotherapie dans les rhumatises chroniques.
Bull. Soc. Med. Hop. Paris. **53**, 323.

Forscher, B.K. (1968).
In: "Chemical Biology of Inflammation". eds. Forscher, B.K. and Houck, J.C. Pergammon Press, London.

Friedman, M. (1977).
Chemical basis for pharmacological and therapeutic actions of penicillamine.
Proc. Roy. Soc. Med. **70**, (supl 3)., 50-60.

Frojmovic, M.M. and Milton, J.G. (1982).
Human platelet size, shape and related functions in health and disease.
Phys. Rev. **62**, 185-261.

Gaarder, A., Jonsen, J., Laland, S., Hellem, A.J. and Owren, P. (1961).
Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets.
Nature (Lond.) **192**, 531-532.

Gaucher, A., Faure, G., Netter, P., Pourel, J. and Duheille, J. (1976).
Contribution of the Scanning Electron Microscope to the study of normal and pathological human synovial membrane.
Rev. Rheum. **43**, 51-60.

Gerber, D.A. (1978).
Inhibition of the denaturation of human gamma globulin by a mixture of D-Penicillamine disulphide and copper.
Biochem. Pharmacol. **27**, 469-472.

Gerrard, J.M., Peterson, D.A. and White, J.G. (1981).
Calcium mobilisation. In: "Platelets in Biology and Pathology". Vol 2. ed. Gordon, J.L. Amsterdam; Elsevier/North Holland Biomedical Press. 407-436.

Gibofsky, A., Winchester, R.J., Pafarroyo, M., Fotino, M. and Kunkel, H.G. (1978).
Disease associations of the IgA-like human alloantigens. Contrasting patterns in rheumatoid arthritis and systemic lupus erythematosus.
J. Exp. Med. **148**, 1728.

Giles, C. (1981).
The platelet count and mean platelet volume.
Br. J. Haematol. **48**, 31-37.

Glynn, L.E. (1972).
Pathology, Pathogenesis of aetiology of rheumatoid arthritis.
Ann. Rheum. Dis. 31, 412-420.

Goldman, J.A., Casey, H.L., McIlwain, H., Kirby, J., Wilson, C.H. and Miller, S.B. (1979).
Limited plasmapheresis in rheumatoid patients with vasculitis.
Arthritis. Rheum. 22, 1146.

Gorog, P., Schraufstatter, I. and Born, G.V.R. (1982).
Effect of removing sialic acids from endothelium on the adherence of circulating platelets in arteries in vivo.
Proc. Roy. Soc. Lond. Biol. Sci. 214, 471-480.

Grant, J.A. and Scrutton, M.C. (1979).
Novel alpha$_2$-adrenoreceptors primarily responsible for inducing human platelet aggregation.
Nature (Lond.) 277, 659-661.

Grant, J.A. and Scrutton, M.C. (1980a).
Interaction of selective alpha adrenoceptor agonists and antagonists with human and rabbit blood platelets.
Br. J. Pharmacol. 71, 121-134.

Grant, J.A. and Scrutton, M.C. (1980b).
Positive interaction between agonists in the aggregation response of human blood platelets: Interaction between ADP and adrenaline and vasopressin.
Brit. J. Haematol. **44**, 109-125.

Greenwald, R.A. (1981).
Effects of oxygen-derived free radicals on connective tissue macromolecules: Inhibition by copper-penicillamine complex.
J.Rheumatol. (Supp 7) **8**, 9-13.

Grennan, D.M., Sloane, D., Behan, A. and Dick, W.C. (1977).
Clinical significance of antibodies to native DNA as measured by a DNA binding technique in patients with articular features of rheumatoid arthritis.
Ann. Rheum. Dis. **36**, 30-33.

Gul, V., Corke, C.F., Huskisson, E.C. and Holborow, E.J. (1984).
The influence of D-penicillamine therapy on numbers of circulating immunoglobulin secreting cells in rheumatoid arthritis.
Rheum. Int. **4**, 157-158.

Hall, N.D. and Bacon, P.A. (1981).
Lymphocytes subpopulations and their role in the rheumatic diseases. In:"Immunological Aspects of Rheumatology". MTP Press, Lancaster. 1-27.

Hall, N.D. and Gillan, A.H. (1979).
Effects of antirheumatic drugs on protein sulphydryl reactivity of human serum.
J. Pharm. Pharmacol. **31**, 676-680.

Hallam, T.J. and Rink, T.J. (1985).
Agonist stimulate divalent cation channels in plasma membrane of human platelets.
FEBS. Lett. **186(2)**, 175-179.

Harker, L.A. (1968).
Kinetics of thrombopoiesis.
J.Clin. Invest. **47**, 458-465.

Harker, L.A. and Finch, C.A. (1969).
Thrombokinetics in man.
J. Clin. Invest. **48**, 963-974.

Harris, E.E. Jr. (1976).
Recent insights into the pathogenesis of the proliferative lesion in rheumatoid arthritis.
Arthritis. Rheum. **19**, 68-72.

Hart, F.D., Huskisson, E.C. and Ansell, B.M. (1982).
Non-steroidal anti-inflammatory analgesics. In: "Drug Treatment of the Rheumatic Diseases". ed. Hart, F.D. Adis-Press. 7-60.

Harvey, W. (1978).
Pathogenesis of rheumatoid arthritis. In: "Rheumatoid Arthritis and Related Conditions". Vol 2. ed. Panayi, G.S. Churchill Livingstone. Edinburgh. 39.

Haslam, R.J. (1973).
Interactions of the pharmacological receptors of blood platelets with adenylate cyclase.
Ser. Haematol. 6, 333-350.

Haslam, R.J. (1975).
Role of cyclic nucleotides in platelet function. In: "Biochemistry and Pharmacology of Platelets." Ciba Foundation Symposium 35, Elsevier / North-Holland Biomedical Press. Amsterdam. 121-151.

Haslam, R.J. and Vanderwel, M. (1982).
Inhibition of platelet adenylate cyclase by 1-O-alkyl-2-O-acetyl-sn-glyceryl-3-phosphorycholine. (Platelet-activating factor).
J. Biol. Chem. 257, 6879-6885.

Hay, F.C., Nineham, L.J., Male, D.K., Roitt, I.M. and Parry, H.(1979a).
Monitoring of plasmapheresis in S.L.E. by immune complex assay. In:"Protides Biological Fluids". ed. Peeters, H. 26, 409.

Hellem, A.J. (1960).
The adhesiveness of human blood platelets in vitro. Scand. J. Clin. Lab. Invest. 12, (Suppl.51): 1-117.

Henson, P.M. (1970).
Release of vasoactive amines from rabbit platelets induced by anti platelet antibody in the presence and absence of complement.
J. Immunol. 104, 924.

Henson, P.M. and Cochrane, C.G. (1969a).
Immunological induction of increased vascular permeability. I. A rabbit passive cutaneous anaphylatic reaction requiring complement, platelets and neutrophils.
J. Exp. Med. 129, 153.

Henson, P.M. and Cochrane, C.G. (1969b).
Immunological induction of increased vascular permeability. II. Two mechanisms of histamine release from rabbit platelets involving complement.
J. Exp. Med. 129, 167.

Heptinstall, S., White, A., Williamson, L and Mitchell, J.R.A. (1985).
Extracts of feverfew inhibit granule secretion in blood platelets and polymorphonuclear leucocytes.
Lancet i, 1071-1074.

Hernandez, L.A., Rowan, R.M., Kennedy, A.C. and Buchanan, W.M. (1975).
Thrombocytosis in rheumatoid arthritis: a clinical study of 200 patients.
Rheumatology 6, 635.

Higgs, G.A. and Youlton, L.J.F. (1972).
Prostaglandin production by rabbit PMN leucocytes in vitro.
Br. J. Pharmacol. 44, 330.

Hildebrandt, J.D., Sekura, R.D., Codina, J., Iyengar, R., Manclark, C.R. and Birnbaumer, L. (1983).
Stimulation and inhibition of adenyl cyclases mediated by distinct regulatory proteins.
Nature (Lond.) 302, 706-709.

Hirata, F., Schiffmann, E., Venkatasubramanian, K., Saloman, D. and Axelrod, J. (1980).
A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticords.
Proc. Natl. Acad. Sci. USA. 77, 2533-2536.

Hjalmarson, O., Hanson, L. and Nibson, L. (1977).
IgA deficiency during D-Penicillamine treatment.
Br.Med.J. 1, 549.

Holborow, E.J. (1979).
Antinucleic acid antibodies.
J. Clin. Pathol. (Suppl) 13, 107-111.

Holmsen, H. (1974).
Are platelet shape change, aggregation and release reaction tangible manifestations of one basic cellular process? In: "Platelets". Production, Function, Transfusion and Storage. eds. Baldini, M.G., and Ebbe, S. Grune & Stratton. 207-220.

Holmsen, H. (1975).
Biochemistry of the platelet release reaction. In: "Biochemistry and Pharmacology of Platelets." Ciba Foundation Symposium. Elsevier, North Holland Biomedical Press. Amsterdam. 175-205.

Holmsen, H. (1976).
Classification and possible mechanisms of action of some drugs that inhibit platelet aggregation.
Ser. Haemat. 8, 50-80.

Holmsen, H. (1977).
Prostaglandin-thromboxane synthesis and secretion as positive feedback loops in the propagation of platelet responses.
Thromb. Haemost. 38, 1030-1041.

Holmsen, H. (1978).
Platelet secretion (Release reaction)- Mechanism and Pharmacology.
Adv. Pharmacol. Therap. 4, 97-109.

Holmsen, H. (1980).
Mechanisms of platelet secretion. In: "Platelets". Cellular response, mechanisms and their biological significance. eds. Rotman, A., Meyer, F.A., Gitler, C., Silberberg, A. John Wiley & Sons Ltd., New York. 249-263.

Holmsen, H. and Karpatkin, S. (1983).
Metabolism of platelets. In: "Haematology" 3rd Edition. eds. Williams, W., Beutler, E. and Erslev. A. New York McGraw-Hill. 1149-1176.

Holmsen, H., Salganicoff, L. and Fukami, M.H. (1977).
Platelet behaviour and biochemistry. In "Biochemistry, Physiology and Pathology of Haemostasis". eds. Ogston, D. and Bennett. B. John Wiley. London. 239-319.

Hopsu-Havu, V.K., Makinen, K.K., Glenner, G.G. (1966).
Formation of bradykinin from Kallidin-10 by aminopeptidase.B.
Nature (Lond.) **212**, 1271.

Houck, J.C. (1968).
In: "Chemical Biology of Inflammation". eds. Houck, J.C. and Forscher, B.F. Pergamon Press. Oxford. 1-3.

Hryszko, S., Pietruska, Z., Bernacka, K. and Bogdanikowa,B. (1975).
Thrombocytosis in rheumatoid arthritis in the light of observation of 150 patients.
Rheumatologia. **13**, 255-261.

Hsu, C.Y., Knapp, D.R. and Halushka, P.V. (1979).
The effects of alpha adrenergic agents on human platelet aggregation.
J. Pharmacol. Exp. Ther. **208**, 366-370.

Huang, E.M. and Detwiler, T.C. (1981).
Characteristics of the synergistic actions of platelet agonists.
Blood **57**, 685-691.

Humes, J.L., Winter, C.A., Sadowski, S.J. and Kuehl, F.A. Jr. (1981).
Multiple sites on prostaglandin cyclo-oxygenase are determinants in the action of non-steroidal anti-inflammatory agents.
Proc. Natl. Acad. Sci. USA. **78**, 2053-2056.

Hurd, E.R. and Ziff, M. (1974).
Parameters of improvement in patients with rheumatoid arthritis treated with cyclophosphamide.
Arthritis. Rheum. **17**, 72.

Hutchinson, R.M., Davis, P. and Jayson, M.I.V. (1976).
Thrombocytosis in rheumatoid arthritis.
Ann. Rheum. Dis. **35**, 138-142.

International Committee for standardization in haematology. (1978).
Recommendations for reference method for haemoglobinometry in human blood and specifications for interternational haemiglobincyanide reference preparation.
J. Clin. Path. **31**, 139.

International Committee on Thrombosis and Haemostasis. (1981).
Recommendations of the international committee on thrombosis and haemostasis concerning platelet membrane glycoprotein nomenclature.
Thromb. Haemost. 46, 764-765.

Israels, E.D., Nisli, G., Paraskevas, F. and Israels, L.G. (1973).
Platelet Fc receptor as a mechanism for Ag-Ab complex induced platelet injury.
Thromb. Diath. Haemorrh. 29, 434-444.

Jaffe, I.A. (1962).
Intra-articular dissociation of the rheumatoid factor.
J. Lab. Clin. Med. 60(3), 409.

Jaffe, I.A. (1963).
Comparison of the effect of plasmapheresis and penicillamine on the level of circulating rheumatoid factor.
Ann. Rheum. Dis. 22, 71-76.

Jaffe, I.A. (1965).
The effect of penicillamine on the laboratory parameters in rheumatoid arthritis.
Arthritis. Rheum. 8, 1064-1079.

Jaffe, I.A. (1979).
Penicillamine in rheumatoid arthritis: clinical pharmacology and biochemical properties.
Scand. J. Rheumatol. **(Suppl.28)**,58-64.

Jakobs, K.H., Lasch, P., Minuth, M., Aktories, K. and Schultz, G. (1982).
Uncoupling of alpha-adrenoceptor mediated inhibition of human platelet adenylate cyclase by N-ethylmaleimide.
J. Biol. Chem. **257**, 2829-2833.

Jakobs, K.H., Saur, W. and Schultz, G. (1976).
Reduction of adenylate cyclase activity in lysates of human platelets by the alpha-adrenergic component of epinephrine.
J. Cylic Nucleotide. Res. **2**, 381-392.

Johnson, P.M. and Faulk, W.P. (1976).
Rheumatoid factor: its nature, specificity and production in rheumatoid arthritis.
Clin. Immunol. Immunopath. **6**, 414-430.

Jones, V.E., Jacoby, R.K., Johnson, P.M., Phua, K.K. and Welsh, K.I. (1983).
Association of HLA-DRW4 with definite rheumatoid arthritis, but not with susceptibility to arthritis.
Ann. Rheum. Dis. **42**, 223.

Jorgensen, L., Hovig, T., Rowsell, H.C. and Mustard, J.F. (1970).
ADP induced platelet aggregation and vascular injury in swine and rabbits.
Am. J. Pathol. 61, 161.-170.

Kaley, G. and Weiner, R. (1971).
Effect of PGE_2 on leucocyte migration.
Nature (New Biol.) 234, 114-115.

Karpatkin, S. (1969a).
Heterogeneity of human platelets. I. Metabolic and kinetic evidence suggestive of young and old platelets.
J. Clin. Invest. 48, 1073-1082.

Karpatkin, S. (1969b).
Heterogeneity of human platelets II. Functional evidence suggestive of young and old platelets.
J. Clin. Invest. 48, 1083-1087.

Karsh, J., Wright, D.G., Klippel, J.H., Decker, J.L., Deisseroth, A.B. and Flye, M.W. (1979).
Lymphocyte depletion by continuous flow cell centrifugation in rheumatoid arthritis.
Arthritis. Rheum. 22, 1055-1059.

Kaufman, R.M., Airo, R., Pollack, S., Crosby, W.H. and Doberneck, R. (1965).
Origin of pulmonary megakaryocytes.
Blood 25, 767-775.

Kendall, M.D. (1977).
Effects of anti-inflammatory drugs on serum chemistry.
J. Int. Med. Res. 5, (Suppl.2) 86-94.

Kerry, R. and Scrutton, M.C. (1983).
Platelet beta-adrenoceptors.
Br. J. Pharmacol. 79, 681-691.

Kinlough-Rathbone, R.L., Cazenave, J.P., Packham, M.A. and Mustard, J.F. (1980).
Effect of inhibitors of the arachidonate pathway on the release of granule contents from rabbit platelets adherent to collagen.
Lab. Invest. 42, 28-34.

Kobayashi, I. and Ziff, M. (1973).
Electron microscopic studies of lymphoid cells in rheumatoid synovial membrane.
Arthritis. Rheum. 16, 471-486.

Kotzin, B.L., Strober, S., Engleman, E.G., Calin, A., Hoppe, R.T., Kansas, G.S., Terrell, C.P. and Kaplan, H.S. (1981).
Treatment of intractable rheumatoid arthritis with total lymphoid irradiation.
New.Eng.J.Med. **305**, 969-976.

Kulka, J.P.D., Bocking, M.W., Ropes, S. and Bayer, W. (1955).
Early joint lesions of rheumatoid arthritis.
Arch. Pathol. **59**, 129-150.

Lam, S.C., Guccione, M.A., Packham, M.A. and Mustard, J.F. (1982).
Effect of cAMP-phosphodiesterase inhibitors on ADP-induced shape change, cAMP and nucleoside diphosphokinase activity of rabbit platelets.
Thromb. Haemost. **47**, 90-95.

Langer, S.Z. (1974).
Presynaptic regulation of catecholamine release.
Biochem. Pharmacol. **23**, 1793-1800.

Langer, S.Z. (1977).
Presynaptic receptors and their role in the regulation of transmitter release.
Br. J. Pharmacol. **60**, 481-497.

Langer, S.Z. (1980).
Presynaptic regulation of the release of catecholamines.
Pharmacol. Rev. **32**, 337-362.

Lapetina, E.G. (1982).
Platelet activating factor stimulates the phosphoinositol cycle.
J. Biol. Chem. **257**, 7314-7317.

Lasch, P. and Jakobs, K.H. (1979).
Agonist and antagonist effects of various alpha adrenergic agonists in human platelets.
Naunyn. Schmiedebergs. Arch. Pharmacol. **306**, 119-125.

Lefkowitz, R.J. (1982).
The Gordon Wilson Lecture.
Adrenergic receptors: regulation at the biochemical physiological and clinical levels.
Trans. Am. Clin. Climatol. Assoc. **94**, 75-90.

Lefkowitz, R.J., Stadel, J.M. and Caron, M.G. (1983).
Adenylate cyclase-coupled beta-adrenergic receptors, structure and mechanisms of activation and desensitization.
Annu. Rev. Biochem. **52**, 159-186.

Leung, L.L.K. and Nachman, R.L. (1982).
Complex formation of platelet thrombospondin with fibrinogen.
J. Clin. Invest. **70**, 542-549.

Leung, N.L., Vickers, J.D., Kinlough-Rathbone, R.L., Reimers, H.J. and Mustard,J.F. (1983b).
ADP-induced changes in (32P) phosphate labelling of phosphatidylinositol-4-5-bisphosphate in washed rabbit platelets made refractory by prior ADP stimulation.
Biochem. Biophys. Res. Commun. **113**, 483-490.

Levin, J. and Bessman, J.D.(1983).
The inverse relation between platelet volume and platelet number.
J. Lab. Clin. Med. **101**, 295-307.

Lewis, D.A. and Day, E.H. (1972).
Biochemical factors in the action of steriods on diseased joints in rheumatoid arthritis.
Ann. Rheum. Dis. **31**, 374.

Lewis, G.P. and Piper, P.J. (1975).
Inhibition of release of prostaglandins as an explanation of some of the actions of anti-inflammatory corticosteroids.
Nature (Lond.) **254**, 308-311.

Lipsky, P.E. (1981).
The effect of D-penicillamine on human helper T cell function in Modulation of Autoimmunity and Disease. In: "The Penicillamine Experience". eds. Maini, R.N. and Berry, H. Praeger. 79-88.

Lipsky, P.E. (1984).
Immunosuppression by D-penicillamine in vitro. Inhibition of human T lymphocyte proliferation by copper-or ceruloplasmin-dependent generation of hydrogen peroxide and protection by monocytes.
J. Clin. Invest. **73**, 53-65.

Lipsky, P.E. and Ziff, M. (1978).
The effect of D-penicillamine on mitogen-induced human lymphocyte proliferation: synergistic inhibition by D-penicillamine and copper salts.
J. Immunol. **120**, 1006-1013.

Lipsky, P.E. and Ziff, M. (1982).
The mechanism of action of gold and D-penicillamine in R.A.
Adv. Inflam. Res. **3**, 219-335.

Ludlam, C.A. (1979).
Evidence for the platelet specificity of beta-thromboglobulin and studies on its plasma concentration in healthy individuals.
Br. J. Haematol. **41**, 271-278.

Ludlam, C.A., Moore, S., Bolton, A.E., Pepper, D.S. and Cash, J.D. (1975).
The release of a human platelet specific protein measured by a radioimmunoassay.
Thromb. Res. **6**, 543-548.

Luscher, E.F. (1971).
Immune complexes and platelet aggregation.
Acta. Med. Scand. **525**, 151.

Luscher, E.F. and Pfueller, S.L. (1978).
Platelets as a target of immunological reactions.
In: "Platelets: A Multi-disciplinary Approach".
eds. de Gaetano, G. and Garattini, S. Raven Press.New York. 261-268.

Lyle, W.H. (1983).
Penicillamine. In "Antirheumatic drugs".
ed. Huskisson, E. Praeger. 518-554.

MacFarlane, D.E., Mills, D.C.B. and Srivastava, P.C. (1982).
Binding of 2-azidoadenosine (beta-32P) diphosphate to the receptor on intact human blood platelets which inhibits adenylate cyclase.
Biochem. 21, 544-549.

Mahadevappa, V.G. and Holub, B.J. (1982).
The molecular species composition of individual diacyl phospholipids in human platelets.
Biochim. Biophys. Acta. 713, 73-79.

Mann, H.B. and Whitney, D.R. (1947).
On a test of whether one of two random variables is stochastically larger than the other.
Ann. Math. Stat. 18, 50-60.

March, R.E., Reeback, J.S., Holborow, E.J., Coombs, R.R.A. (1981).
MRSPAH: A simple microtitre plate test for rheumatoid factors of different classes.
J. Immuno. Methods. 42, 137-146.

Marcus, A.J. (1978).
The role of lipids in platelet function: with particular reference to the arachidonic acid pathway.
J. Lipid. Res. 19, 793-826.

Margaretten, W. and McKay, D.G. (1971).
The requirement for platelets in the active Arthus reaction.
Am. J. Pathol. **64**, 257.

Mbuyi-Muamba, J., Stevens, E. and Decqueker, J. (1981).
Good response to D-penicillamine in IgA deficient rheumatoid arthritis.
Scand. J. Rheumatol. **10**, 31-32.

Mills, D.C.B., Robb, I.A. and Roberts, G.C.K. (1968).
The release of nucleotides, 5-hydroxytryptamine and enzymes from human blood platelets during aggregation.
J. Physiol. (Lond.) **195**, 715-729.

Mills, D.C.B. and Roberts, G.C.K. (1967).
Effects of adrenaline on human blood platelets.
J. Physiol. (Lond.) **193**, 443-453.

Moncada, S. and Vane, J.R. (1979).
Arachidonic acid metabolites and the interaction between platelets and blood vessel walls.
New. Eng. J. Med. **300**, 1142-1147.

Moore, S., Pepper, D.S. and Cash, J.D. (1975).
The isolation and characterisation of a platelet specific beta-globulin (B-thromboglobulin) and the detection of antiurokinase and antiplasmin released from thrombin-aggregated washed human platelets. Biochim. Biophys. Acta. **379**, 360-369.

Moore, S. and Pepper, D.S. (1976).
Identification and characterisation of a platelet specific release product: B-thromboglobulin. In: "Platelets in Biology and Pathology". ed. Gordon, J.L. Amsterdam, Elsevier/North-Holland Biomedical Press. 293-311.

Morgan, J.E., Hall, N.D., Collins, A.J. and Bacon, P.A. (1980).
The nonspecific inhibitory effect of synovial tissue extracts on leucocyte migration in vitro. Ann. Rheum. Dis. **39**, 323-328.

Motulsky, H.J. and Insel, P.A. (1982).
(^{3}H) Dihydroergocryptine binding to alpha-adrenergic receptors of human platelets. A reassessment using the selective radioligands (^{3}H) prazosin, (^{3}H) yohimbine, and (^{3}H) rauwolscine. Biochem. Pharmacol. **31**, 2591-2597.

Multicentre Trial Group. (1973).
Controlled trial of D-penicillamine in severe rheumatoid arthritis.
Lancet 1, 275-280.

Mueller-Eckardt, C. and Luscher, E.F. (1968).
Immune reactions of human blood platelets. A comparative study on the effects on platelets of heterologous antiplatelet antiserum, antigen-antibody complexes, aggregated gammaglobulin and thrombin.
Thromb. Diath. Haemorrh. 20, 155-167.

Munthe, E., Jellum, E. and Aaseth, J. (1979).
Some aspects of the mechanism of action of penicillamine in rheumatoid arthritis.
Scand. J. Rheumatol. (Suppl. 28) 6-12.

Mustard, J.F., Packham, M.A., Perry, D.W., Guccione, M.A. and Kinlough-Rathbone, R.L. (1975).
In: "Biochemistry and Pharmacology of Platelets." Ciba. Foundation Symposium, 35, Elsevier/North Holland. New York. 47-50.

Mustard, J.F., Kinlough-Rathbone, R.L., Packham, M.A., Perry, D.W., Harfenist, E.F. and Pai, K.R.M. (1979). Comparison of fibrinogen association with normal and thrombasthenic platelets on exposure to ADP or chymotrypsin.
Blood **54**, 987-993.

Nachman, R.L. (1978).
The platelet as an inflammatory cell. In: "Platelets". A Multidisciplinary Approach. eds. de Gaetano, G. and Garattini, S. Raven Press. New York. 199.

Nachman, R.L. and Ferris, B. (1968).
Studies on human platelet protease activity.
J. Clin. Invest. **47**, 2530-2540.

Nachmias, V.T. (1980).
Cytoskeleton of human platelets at rest and after spreading.
J. Cell. Biol. **86**, 795-802.

Nahorski, S.R. (1982).
Alpha and beta-adrenoceptor coupling to adenylate cyclase.
Biochem. Soc. Trans. **10**, 498-500.

Natvig, J.B. and Munthe, E. (1975).
Self-associating IgG rheumatoid factor represents a major response of plasma cells in rheumatoid inflammatory tissue.
Ann. N. Y. Acad. Sci. **256**, 88-95.

Ng, K.C., Brown, K.A., Perry, J.D. and Holborow, E.J. (1980).
Anti-RANA antibody: a marker for seronegative and seropositive rheumatoid arthritis.
Lancet **i**, 447-449.

Nickander, R., McMahon, F.G. and Ridolfo, A.S. (1979).
Nonsteroidal anti-inflammatory agents.
Ann. Rev. Pharmacol. Toxicol. **19**, 469-490.

Nimni, M.E. (1977).
Mechanism of inhibition of collagen cross linking by penicillamine.
Proc. Roy. Soc. Med. **70**, (Suppl.3), 65-72.

Nineham, L.J., Hay, F.C., Male, D.K., Roitt, I.M., Young, A. and Perumal, R. (1979).
Immune-complexes in rheumatoid arthritis: correlations with clinical features and effects of gold.
Protides of the Biological Fluids. **26**, 179.

Norval, M. and Smith, C. (1979).
Search for viral nucleic sequences in rheumatoid cells.
Ann. Rheum. Dis. **38**, 456-462.

Nurden, A.T. and Caen, J.P. (1976).
Role of surface glycoproteins in human platelet function.
Thromb. Haemost. **35**, 139-150.

Nyman, D. (1977).
Collagen induced platelet aggregation. Evidence of several mechanisms for the induction of platelet release by collagen.
Thromb. Res. **10**, 743-751.

O'Brien, J.R. (1963).
Some effects of adrenaline and anti-adrenaline compounds on platelets in vitro and in vivo.
Nature (Lond.) **200**, 763-764.

O'Brien, J.R. (1964a).
A comparison of platelet aggregation produced by seven compounds and a comparison of their inhibitors.
J. Clin. Pathol. **17**, 275-281.

O'Brien, J.R. (1964b).
Variability in aggregation of human platelets by adrenaline.
Nature (Lond.) 202, 1188-1190.

O'Brien, J.R. (1978).
Exhausted platelets continue to circulate.
Lancet ii, 1316-1317.

Owen, J.S., Hutton, R.A., Day,R.C., Bruckdorfer, K.R. and McIntyre, N. (1981).
Platelet lipid compostition and platelet aggregation in human liver disease.
J. Lipid. Res. 22, 423-430.

Owen, N.E., Feinberg, H. and Le Breton, G.C. (1980).
Epinephrine induces Ca^{2+} uptake in human blood platelets.
Am. J. Physiol. 239, H483-H488.

Owen, N.E. and Le Breton, G.C. (1980).
The involvement of calcium in epinephrine or ADP potentiation of human platelet aggregation.
Thromb. Res. 17, 855-863.

Owen, N.E. and Le Breton, G.C. (1981).
Ca^{2+} mobilization in blood platelets as visualised by chlortetracycline fluorescence.
Am. J. Physiol. **241**, H613-H619.

Packham, M.A., Guccione, M.A., Chang, P.L. and Mustard, J.F. (1973).
Platelet aggregation and release effects of low concentrations of thrombin or collagen.
Am. J. Physiol. **225**, 38-47.

Panayi, G.S. and Wooley, P.H. (1977).
B Lymphocyte alloantigens in the study of the genetic basis of rheumatoid arthritis.
Ann. Rheum. Dis. **36**, 365-368.

Pareti, F.I., Capitanio, A. and Mannucci, P.M. (1976).
Acquired storage pool disease in platelets during disseminated intravascular coagulation.
Blood **48**, 511-515.

Parrillo, J.E. and Fauci, A.S. (1979).
Mechanisms of glucocorticoid action on immune processes.
Ann. Rev. Pharmacol. Toxicol. **19**, 179-201.

Paulus, J.M., Breton-Gorius, J., Kinet-Denoel, C. and Boniver, J. (1974).
Megakaryocyte ultrastructure and ploidy in macrothromboytosis. In: "Platelets". Production, Function, Transfusion and Storage. eds. Baldini, M.G. and Ebbe, S. New York. Grune and Stratton. 131-141.

Paulus, H.E., Machleder, H.I., Levine, S., Yu, D.T.Y. and MacDonald, N.S. (1977).
Lymphocyte involvement in rheumatoid arthritis. Studies during thoracic duct drainage.
Arthritis. Rheum. **20**, 1249-1262.

Pazdur, J. and Kopec, M. (1970).
Platelets in rheumatoid arthritis.
Thromb. Diath. Haemorrh. **23**, 276-285.

Peerschke, E.I. (1982).
Induction of human platelet fibrinogen receptors by epinephrine in the absence of released ADP.
Blood **60**, 71-77.

Pegels, J., Ament, H., Van Der Plass Den Dalen, C., Van Dem Borne, A. and Feltkamp, T. (1982).
Autoimmune thrombocytopenia induced by D-penicillamine. In: "Immunogenetics in Rheumatology". (Musculoskeletal disease and penicillamine). eds. Dawkins, R., Christiansen, F. and Zilko, P. Excerpta. Medica. 344-349.

Pekoe, G., Van Dyke, K., Mengoli, H., Peden, D. and English. D. (1982).
Comparison of the effects of anti-oxidant non-steroidal anti-inflammatory drugs against myelo-peroxidase and hypochlorous acid luminol-enhanced chemiluminescence.
Agents. Actions. 12, 232.

Penington, D.G., Streatfield, K. and Roxburgh, A.E. (1976).
Megakaryocytes and the heterogeneity of ciculating platelets.
Br. J. Haematol. 34, 639-653.

Pepper, D.S. and Ludlam, C.A. (1977).
B-thromboglobulin (B.T.G.) a new approach to the diagnosis of Thrombosis. In: "Platelets and Thrombosis". eds. Mills, D.C.B. and Pareti, F.I. New York, Academic Press.

Pfueller, S.L. and Cosgrove, L.J. (1980).
Activation of human platelets in PRP via their Fc receptor by antigen-antibody complexes, or immunoglobulin G: antigen requirement for particle-bound fibrinogen.
Thromb. Res. **20**, 97-108.

Pfueller, S.L. and Luscher, E.F. (1972).
The effects of aggregated immunoglobulins on human blood platelets in relation to their complement-fixing abilities.I. Studies of immunoglobulins of different types.
J. Immunol. **109**, 517-525.

Pfueller, S.L. and Luscher, E.F. (1974).
Studies on the mechanisms of the human platelet release reaction induced by immunological stimuli.
J.Immunol. **112**, 1201-1210.

Phillips, D.R. and Baughan, A.K. (1983).
Fibrinogen binding to human platelet plasma membranes. Identification of two steps requiring divalent cations.
J. Biol. Chem. **258**, 10240-10246.

Pinckard, R.N., McManus, L.M. and Hanahan, D.J. (1982).
Chemistry and biology of acetyl-glyceryl-ether-phosphorylcholine (Platelet-Activating Factor).
Adv. Inflam. Res. 4, 147-180.

Prescott, S.M. and Majerus, P.W. (1983).
Characterization of 1-2-diacylglycerol hydrolsis in human platelets. Demonstration of an arachidonoyl-monoacylglycerol intermediate.
J. Biol. Chem. 258, 764-769.

Pritchard, M. amd Nuki, G. (1978).
Gold and penicillamine: A proposed mode of action in rheumatoid arthritis, based on synovial fluid analysis.
Ann. Rheum. Dis. 37, 493-503.

Raines, E.W. and Ross, R. (1982).
Platelet-derived growth facter 1. High yield and purification and evidence for multiple forms.
J. Biol. Chem. 257, 5154-5160.

Rao, G.H.R., Reddy, K.R. and White, J.G. (1981).
Modification of human platelet response to sodium arachidonate by membrane modulation.
Prostaglandins Leukotrienes Med. 6, 75-90.

Rao, G.H.R. and White, J.G. (1981).
Epinephrine potentiation of arachidonate induced aggregation of cyclooxygenase-deficient platelets.
Am. J. Haematol. **11**, 355-366.

Rittenhouse, S.E. (1982).
Inositol lipid metabolism in the responses of stimulated platelets.
Cell. Calcium. **3**, 311-322.

Rittenhouse-Simmons, S.E. and Deykin. D. (1981).
Release and metabolism of arachidonate acid in human platelets. In: "Platelets in Biology and Pathology". Vol.2. ed. Gordon, J.L. Amsterdam: Elsevier/North-Holland Biomedical Press. 349-372.

Rodbell, M. (1980).
The role of hormone receptors and GTP-regulatory proteins in membrane transduction.
Nature (Lond.) **284**, 17-22.

Rose, H.M., Ragan, C., Pearce, E. and Lipman, M.O. (1948).
Differential agglutination of normal and sensitized sheep erythrocytes by sera of patients with rheumatoid arthritis.
Proc. Soc. Exp. Biol. Med. **68**, 1-6.

Ross, R., and Vogel, A. (1978).
The platelet-derived growth factor: Review.
Cell. **14**, 203-210.

Ruddy, S. and Austen, K.F. (1975).
Activation of the complement and properdin systems in rheumatoid arthritis.
Ann. N.Y. Acad. Sci. **256**, 96.

Sakariassen, K.S., Bolhuis, P.A. and Sixma, J.J. (1979).
Adhesion of human blood platelets to human artery subendothelium is mediated by F VIII -VWF bound to subendothelium.
Nature (Lond.) **279**, 636-638.

Sanders, V.M. and Munson, A.E. (1985).
Norepinephrine and the antibody response.
Pharmacol. Rev. **37**, 229-248.

Sarkar, B., Sass-Kortsak, A., Clarke, R., Laurie, S.H. and Wei, P. (1977).
A comparative study of in vitro and in vivo interaction of D-penicillamine and triethylenetetramine with copper.
Proc. Roy. Soc. Med. **70**, (Suppl. 3), 13-18.

Scatchard, G. (1949).
The attractions of proteins for small molecules and ions.
Ann. N.Y. Acad. Sci. **51**, 660-672.

Schlosstein, L., Terasaki, P.I., Bluestone, R. and Pearson, C.M. (1973).
High association of an HL-A antigen, W27, with ankylosing spondylitis.
New. Eng. J. Med. **288**, 704-706.

Schumacher, H.R. (1975).
Synovial membrane and fluid morphological alterations in early rheumatoid arthritis.
Annals. N. Y. Acad. Sci. **256**, 39-64.

Scrutton, M.C. and Wallis, R.B. (1981).
Catecholamine receptors. In: "Platelets in Biology and Pathology". Vol 2. ed. Gordon, J.L. Elsevier, North Holland Biomedical Press. 179-210.

Selroos, O. (1972).
Thrombocytosis in rheumatoid arthritis.
Scand. J. Rheum. **1**, 136-140.

Selroos, O., and Wegelius, O. (1973b).
Thrombocytes in fibrinogen induced arthritis in rabbbits.
Scand. J. Rheum. **2**, 167-172.

Selroos, O., Petterson, T. and Wegelius, O. (1973c).
Thrombocytosis in experimental amyloidosis in rabbits.
Scand. J. Rheum. **2**, 183.

Shattil, S.J., McDonough, M., Turnbull, J. and Insel, P.A. (1981).
Characterization of alpha adrenergic receptors in human platelets using (^{3}H) colonidine.
Mol. Pharmacol. **19**, 179-183.

Sigler, J.W., Bluhm, G.B., Duncan, H., Sharp, J.T., Ensign, D.C. and McCrum, W.R. (1974).
Gold salts in the treatment of rheumatoid arthritis: A double-blind study.
Ann. Intern. Med. **80**, 21.

Siminovitch, L., McCulloch, E.A. and Till, J.E. (1963).
The distribution of colony-forming cells among spleen colonies.
J. Cell. Comp. Phys. **62**, 327-336.

Sixma, J.J. (1981b).
The haemostatic plug. In: "Recent Advances in Blood Coagulation". Vol.3. ed. Poller, L. London. Churchill Livingstone. 175-192.

Slavin, S. and Strober, S. (1981).
In vitro T cell mediated function in patients with active rheumatoid arthritis.
Ann. Rheum. Dis. **40**, 60-63.

Smiley, J.D., Sachs, C. and Ziff, M. (1968).
In Vitro synthesis of immunoglobulin by rheumatoid synovial membrane.
J. Clin. Invest. **47**, 624-632.

Smith, A.F. and Castor, C.W. (1978).
Connective tissue activation. XII. Platelet abnormalaties in patients with rheumatoid arthritis.
J. Rheum. **5**, 177-183.

Smith, J.B. and Willis, A.L. (1971).
Aspirin selectively inhibits prostaglandin production in human platelets.
Nature (Lond.) **231**, 235-237.

Spector, W.G. and Willoughby, D.A. (1968).
"The Pharmacology of Inflammation".
English Universities Press. London.

Staite, N., Zoschke, D. and Messner, R. (1984).
Scavenging of hydrogen peroxide - a new mechanism of action for D-penicillamine in rheumatoid arthritis.
New. Eng. J. Med. **311(8)**, 538-539.

Stanworth, D.R. (1984).
D-penicillamine in: "Textbook of Immuno-pharmacology". eds. Dale, M. and Foreman, J.C. Blackwell Scientific Publications. 371-380

Stanworth, D.R. and Hunneyball, I.M. (1979).
Influence of D-penicillamine treatment on the humoral immune system.
Scand. J. Rheum. **Suppl.(28)**, 37-46.

Stanworth, D.R., Johns, P., Williamson, N., Shadforth, M., Felix-Davies, D. and Thompson, R. (1977).
Drug induced IgA deficiency in rheumatoid arthritis.
Lancet **i**, 1001-1002.

Starke, K. (1977).
Regulation of noradrenaline release by presynaptic receptor systems.
Rev. Physiol. Biochem. Pharmacol. **77**, 1-124.

Starke, K. (1981).
Alpha-adrenoceptor subclassification.
Rev. Physiol. Biochem. Pharmacol. **88**, 199-236.

Starke, K., Endo, T. and Taube, H.D. (1975).
Relative pre-and postsynaptic potencies of alpha-adrenoceptors agonists in the rabbit pulmonary artery.
Naunyn-Schmiedebergs Arch. Pharmacol. **291**, 55-78.

Stastny, P. (1978).
Association of the B-cell alloantigen DRW4 with rheumatoid arthritis.
New. Eng. J. Med. **298**, 869-871.

Stastny, P. and Fink, C.W. (1979).
Different HLA-D associations in adult and juvenile rheumatoid arthritis.
J. Clin. Invest. **63**, 124-130.

Steffen, C. (1980).
Collagen as a antiantigen in rheumatoid arthritis.
In: "Studies in Joint Disease". eds. Maroudas, A. and Holborow, E.J. Pitman Medical Ltd., Kent. 201-226.

Strober, S. (1981).
Immunologic aspects of inflammation, lymphocyte populations. In: "Textbook of Rheumatology". Vol 1, eds. Kelly, W.N., Harris, E.D., Ruddy, S. and Sledge, C.B., Saunders Company. Philadelphia. 19-30.

Swinson, D.R. and Swinburn, W.R. (1980).
eds. Rheumatology. Hodder and Stoughton Educational. Suffolk.

Takayama, H., Okuma, M. and Uchino, H. (1980).
A simple method for estimation of lipoxygenase and cyclo-oxygenase pathways in human platelets - the use of thiobarbituric acid reaction.
Thrombo. Haemost. 44, 111-114.

Tenenbaum, J., Urowitz, M.B., Keystone, E.C., Dwosa, I.L. and Curtis, J.E. (1979).
Leucapheresis in severe rheumatoid arthritis.
Ann. Rheum. Dis. 38, 40-44.

Thomas, D., Gallus, A.S., Brooks, P.M., Tampi, R., Geddes, R. and Hill, W. (1984).
Thrombokinetics in patients with rheumatoid arthritis treated with D-penicillamine.
Ann. Rheum. Dis. 43, 402-406.

Till, J.E. and McCulloch, E.A. (1961).
A direct measurement of the radiation sensitivity of normal mouse bone marrow cells.
Radiation Research. 14, 213-222.

Tracy, P.B. and Mann, K.G. (1983).
Prothrombinase complex assembly on the platelet surface is mediated through the 74,000 dalton component factor Va.
Proc. Natl. Acad. Sci. USA. **80**, 2380-2384.

U'Prichard, D.C., Mitrius, J.C., Kahn, D.J. and Perry, B.D. (1982).
The alpha - adrenergic receptor: multiple affinity states and regulation of a receptor inversely coupled to adenylate cyclase, In: "The Molecular Pharmacology of Neurotransmitter Receptor Systems". eds. Segawa, T. and Yamamura, H.C. Raven Press, New York.

Valenti, G., Chianese, U., Tirri, G. and Giordono, M. (1979).
Thrombocytosis as an inflammation index in rheumatic and connective tissue diseases. In: "Advances in Inflammation Research". eds. Weissman, G., Samuelsson, B and Paoletti, R. Raven Press, New York. 579.

Vane, J.R. (1971).
Inhibition of prostaglandin synthesis as a mechanism of action for aspirin - like drugs.
Nature (New Biol.) **231**, 232-235.

Verrier Jones, J., Cumming, R.H., Bacon, P.A., Evers, J., Fraser, I.D., Bothamley, J., Tribe, C.R., Davis, P. and Hughes, G.R. (1979).
Evidence for a therapeutic effect of plasmapheresis in patients with system Lupus Erythematosus.
Quart. J. Med. **48**, 555.

Vollersten, R.S., Fuster, V., Conn, D.L., Luthra, H.S., McDuffie, F.C., Bowie, E.J.W. and Ilstrup, D.M. (1982).
In vivo platelet survival in rheumatoid arthritis.
Mayo. Clin. Proc. **57**, 620-624.

Waaler, E. (1940).
On the occurrence of a factor in human serum activating the specific agglutination of sheep blood corpuscles.
Acta. Path. Microbiol. Scand. **17**, 172-188.

Walshe, J.M. (1956).
Wilson's Disease. New Oral therapy.
Lancet **i**, 25-26.

Walz, D.T., DiMartino, M.J. and Sutton, B.M. (1974).
Design and laboratory evaluation of gold compounds on anti-inflammatory agents.
Med. Chem. **13**, 209.

Weiner, M. and Piliero, S.J. (1970).
Non-steriod anti-inflammatory agents.
Ann. Rev. Pharmacol. **10**, 171-198.

Weissmann, G. (1967).
The role of lysosomes in inflammation and disease.
Ann. Rev. Med. **18**, 97-112.

Weissmann, G. (1972).
Lysosomal mechanisms of tissue injury in arthritis.
New. Eng. J. Med. **286**, 141-146.

Weksler, B.B. amd Coupal, C.E. (1973).
Platelet dependent generation of chemotactic activity in serum.
J. Exp. Med. **137**, 1419-1430.

Werner, G. and Morgenstern, E. (1980).
Three dimensional reconstruction of human blood platelets using serial sections.
Eur. J. Cell Biol. **20**, 276-282.

Westwick, J., Poll, C. and Kakkar, V.V. (1984).
IUPHAR 9th International congress on Pharmacology.
eds. Paton, W., Mitchell, J. and Turner, P. London.
MacMillan. (Abst.) 178.

White, J.G. (1979).
Current concepts of platelet structure.
Am. J. Clin. Path. **71**, 363-378.

White, J.G., Clawson, C.C. and Gerrard, J.M. (1981).
Platelet ultrastructure. In: "Haemostasis and Thrombosis". eds. Bloom, A.L. and Thomas, D.P. London. Churchill Livingstone. 22-49.

White, J.G. and Gerrard, J.M. (1982).
Anatomy and structural organisation of the platelet. In: "Haemostasis and Thrombosis. Basic Principals and Clinical Practice". eds. Colman, R.W., Hirsh, J., Marder, V.J. and Salzman, E.W. Philadelphia-Toronto. J.B. Lippincott. 343-363.

Wiggins, R.C. and Cochrane, C.G. (1981).
Immune-complex-mediated biologic effects.
New. Eng. J. Med. **304**, 518-520.

Willoughby, D.A. (1973).
The control of the inflammatory response with special reference to the prostaglandins. In: "Prostaglandins and Cyclic AMP- Biological Actions and Clinical Applications". eds.Kahn, R.H. and Lands, W.E.M. Academic Press New York and London.

Winchester, R.J., Agnello, V. and Kunkel, H.G. (1969).
An association between the gamma G complexes and complement depletion in joint fluids of patients with rheumatoid arthritis.
Arthritis. Rheum. 12, 343.

Winchester, R.J., Agnello, V. and Kunkel, H.G. (1970).
Gamma globulin complexes in synovial fluids of patients with rheumatoid arthritis. Partial characterization and relationship to lowered complement levels.
Clin. Exp. Immunol. 6, 689.

Wollheim, F.A. (1981).
Effects of D-penicillamine on circulating protein complexes in rheumatoid arthritis and primary biliary cirrhosis.
J. Rheumatol. (Suppl.7) 8, 74-79.

Wood, P.H.N. (1977).
ed. In: "The Challenge of Arthritis and Rheumatism".
The British league against Rheumatism.

Yokoyama, M., Kawishima, S., Sakamoto, S., Akita, H., Okada, T., Mizutani, T. and Fukuzaki, H. (1983). Platelet reactivity and its dependence on alpha-adrenergic receptor function in patients with ischaemic heart disease. Br. Heart. J. **49**, 20-25.

Yu, S.K. and Latour, J.G. (1977). Potentiation by alpha and inhibition by beta-adrenergic stimulations of rat platelet aggregation. A comparative study, with human and rabbit platelets. Thromb. Haemost. **37**, 413-422.

Zahavi, J. and Kakkar, V.V. (1980). Beta-thromboglobulin a specific marker of in-vivo platelet release reaction. Thromb. Haemost. **44**, 23-29.

Zavoico, G.B., and Feinstein, M.B. (1984). Cytoplasmic Ca^{2+} in platelets is controlled by cyclic AMP: antagonism between stimulators and inhibitors of adenylate cyclase. Biochem. Biophys. Res. Commun. **120**, 579-585.

Ziff, M. (1980).

Future trends in the Immunopathology of Rheumatoid Arthritis. In: "Inflammation". Mechanisms and treatment. eds. Willoughby, D.A. and Giroud, J.P. M.T.P Press Ltd., Lancaster. 43-52.

Zimmerman, T.S. and Kolb, W.P. (1976).

Human platelet initiated formation and uptake of the C5-9 complex of human complement.
J. Clin. Invest. **57**, 203-211.

Zvaifler, N.J. (1973).

The immunopathology of joint inflammation in rheumatoid arthritis.
Adv. Immunol. **16**, 265-336.

Zvaifler, N.J. (1974).

Rheumatoid synovitis. An extravascular immune complex disease.
Arthritis. Rheum. **17**, 297.

Zvaifler, N.J. (1979a).

Etiology and Pathogenesis of Rheumatoid Arthritis. In: "Arthritis and allied conditions". 9th Edition ed. McCarty, D.J., Lea and Fabiger, Philadelphia. 417-428.

Zvaifler, N.J. (1979b).
Gold and antimalarial therapy. In: "Arthritis and Allied Conditions". 9th Edition ed. McCarty, D.J., Lea and Fabiger, Philadelphia. 355-367.

Zweifach, B.W., Grant, L. and McCluskey, R.T. (1965).
eds. "The Inflammatory Process". Academic Press. New York and London.

ADDENDUM

Wallace, S.L. and Ragan, C. (1958).
The problem of therapeutic evaluation in Rheumatoid Arthritis.
Arthritis. Rheum. I, 20-28.